Vol. XLV No. 4

April 1956

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

Two Cyclic Analogs of Acetylcholine 197
F. W. Schueler

The Chemical Evaluation of *Rauwolfia serpentina* Preparations 200
Jonas Carol, Daniel Banes, Jacob Wolff, and *Herman O. Fallscheer*

The Effect of Various Preservatives on Microorganisms Isolated from Deteriorated Syrups 204
Jerome Schimmel and *William J. Husa*

An Investigational Study of Hydroxy Aluminum Magnesium Aminoacetate, A New Antacid 208
Arthur G. Zupko

Modern Ointment Base Technology II. Comparative Evaluation of Bases 212
Margaret N. Mutimer, Charles Riffkin, John A. Hill, Murray E. Glickman, and *Gilman N. Cyr*

1-Phenyl-2-Amino-3-Methoxy-1-Propanol Hydrochloride and Related Compounds 219
Jekishan R. Parikh and *J. F. Oneto*

Anticoagulant Sulfuric Acid Esters of Synthetic Polyglucoses 223
Dale P. J. Goldsmith, Kane L. Kelley, and *Charles W. Mushett*

A Pharmacologic Study of Combinations of Antihistaminic Substances 225
Joseph A. Kaiser and *Casimir T. Ichniowski*

A Paper Chromatographic Study of Aloe, Aloin and of Cascara Sagrada 229
Nouri Y. Mary, Bernard V. Christensen, and *Jack L. Beal*

A Simplified Assay of Nux Vomica Tincture 232
Morton Scott, Abraham Taub, and *Claude Piantadosi*

The Toxic Component of the Singletary Pea (*Lathyrus pusillus*) 236
Harold P. Dupuy and *Jordan G. Lee*

Ultrasonic Extraction of *Cinchona succirubra* 239
W. F. Head, Jr., H. M. Beal, and *W. H. Lauter*

Extraction of *Cinchona succirubra* with Tetrahydrofuran and its Mixtures 242
J. M. Campo and *L. G. Gramling*

The "Melting-Point" of Suppositories. Device for Testing the Resistance of Fatty-Base Suppositories to Deformation 246
S. Reznek

Digitalis Studies by the U. S. P. XIV Method of Assay I. Seasonal Variation in Response of the Pigeon to Digitalis 248
R. A. Sachs, J. D. Highstrete, and *M. L. Pabst*

Digitalis Studies by the U. S. P. XIV Method of Assay II. Stability of Crude Digitalis Leaf 250
R. A. Sachs, J. D. Highstrete, and *M. L. Pabst*

Sabadilla Alkaloids V. Vanilloylcevine 252
David M. Stuart and *Lloyd M. Parks*

A New Approach to the Chemistry of Aromatic Ammonia Spirit 257
Willis E. Moore and *David Abraham*

p-Hydroxybenzoic Acid Esters as Preservatives II. Acute and Chronic Toxicity in Dogs, Rats, and Mice 260
Carol Matthews, Joan Davidson, Elizabeth Bauer, James L. Morrison, and *Arthur P. Richardson*

p-Hydroxybenoic Acid Esters as Preservatives III. The Physiological Disposition of *p*-Hydroxybenzoic Acid and Its Esters 268
Passie S. Jones, Doris Thigpen, James L. Morrison, and *Arthur P. Richardson*

A Note on the Determination of Some Alkali Salts of Organic Acids by Ion Exchange 274
Seymour M. Blaug

Book Notices 275

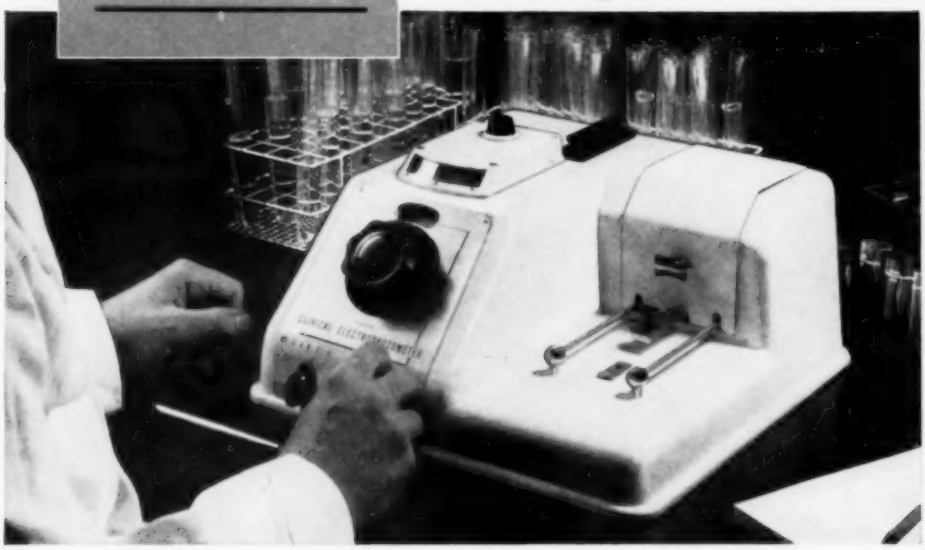
New and Nonofficial Remedies ..... III, IV, V

Advertising ..... Cover pages 2, 3, 4, and V–VIII

© *Copyright 1956, by the American Pharmaceutical Association*

Rapid, Accurate Colorimetric Analyses with the NEW

CLINICAL electrophotometer®

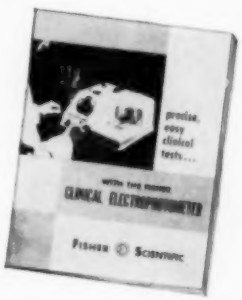


The Fisher CLINICAL Electrophotometer is a colorimetric instrument for the *rapid* and *accurate* quantitative determination of constituents of biological fluids. It is the essential instrument for chemical and biochemical colorimetric analyses.

This brand new instrument was developed around the successful background of the Fisher Electrophotometer, the widely accepted instrument for colorimetry in the chemical field.

It is line-operated (no batteries required) and uses a balanced circuit which compensates for line voltage fluctuations and other variables.

It is extremely versatile and very simple and convenient to operate. A very complete manual is included with the CLINICAL Electrophotometer which provides easy-to-follow instructions for operation of the instrument, *a wide variety of methods*, and lists of solutions and apparatus required for each method described.



WRITE FOR THIS FREE BOOKLET→

125 Fisher Bldg., Pittsburgh 19, Pa.

FISHER SCIENTIFIC

Boston, Buffalo, Chicago, Cleveland, Detroit, New York, Philadelphia, Pittsburgh, St. Louis, Washington, Montreal, Toronto

America's Largest Manufacturer-Distributor of Laboratory Appliances and Reagent Chemicals

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

SCIENTIFIC EDITION

Committee on Publications

Don E. Francke, *Chairman*
Robert P. Fischelis
John B. Heinz
R. Q. Richards
Hugo H. Schaefer

Editorial Advisory Board

John E. Christian
Walter C. Gakenheimer
George P. Hager
Ewart A. Swinyard
Elmer B. Vliet
H. W. Youngken, Jr.

JUSTIN L. POWERS, EDITOR
SAMUEL W. GOLDSTEIN, ASSISTANT EDITOR
ROBERT I. BISCHOFF, ADVERTISING MANAGER

DRUG STANDARDS

A Publication of The American Pharmaceutical Association

Single copies $1.00
Annual Subscription $5.00

Order from

The American Pharmaceutical Association

2215 Constitution Avenue Washington 7, D. C.

The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

Editorial and Executive Offices—2215 Constitution Avenue, N.W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition and Scientific Edition combined: United States, Canada, Pan America, Spain, and Philippines, $10.00; other foreign, $12.00.

Journal of the American Pharmaceutical Association, Scientific Edition alone: United States, Canada, Pan America, Spain, and Philippines, $6.00; other foreign, $7.00.

Journal of the American Pharmaceutical Association, Practical Pharmacy Edition alone: United States, Canada, Pan America, Spain, and Philippines, $5.00; other foreign, $6.00.

Members of the American Pharmaceutical Association receive the combined subscription as a part of their annual dues. Members may request omission of either edition, but this will not reduce the amount of the annual dues.

Single Copies—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition: United States, Canada, Pan America, Spain, and Philippines, $0.50; other foreign, $0.60. Scientific Edition: United States, Canada, Pan America, Spain, and Philippines, $0.60; other foreign, $0.70.

Back issues of unbound Journals same price as current volume.

Subscription rates are subject to change without notice.

Change of Address—Members and subscribers should bear in mind that second class matter is not forwarded by the Post Office and should supply both the Post Office and the American Pharmaceutical Association with any change of address immediately upon its occurrence.

Claims for missing numbers will not be allowed if received more than 60 days after the date of issue, or if loss was due to failure to give 30 days' notice of change of address. Claims asserting that Journals are "missing from files" cannot be allowed. The Association cannot accept responsibility for foreign delivery when its records indicate that shipment has been made.

Entered as second class matter January 23, 1917, at the Post Office at Easton, Pennsylvania, under the Act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 200 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

References to books should be in the order given, (1) author's surname and initials, (2) title of the book, (3) volume, (4) edition, (5) name of publisher, (6) place of publication, (7) year of publication, and (8) page number. A complete book reference may be illustrated as follows:

(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages, and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e. g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mm., cm., and L.

Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

Numbers and measurements, etc., which are to appear on the axes of the drawing should be indicated in pencil outside the axes, in order that they may be set in type. Captions and legends should be placed *below* the diagram (not on it), or, if lengthy, collected on a separate typewritten list, identifying each by its proper figure number and including whatever acknowledgments are necessary.

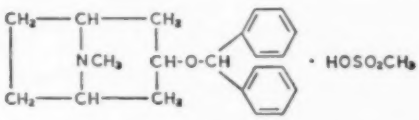
Photographs should never be rolled, and paper clips should not be used unless guarded by a piece of paper folded several times to prevent an indentation in the photograph. All drawings and photographs should be protected from bending and from the chance of having their edges torn while in transit. Do not write on the back of a photograph. Any indentation in the photograph, however slight, will be likely to show as a blemish in the reproduction. All figures submitted must be referred to in the text of the manuscript, and should be numbered consecutively with Arabic numerals, e. g., Fig. 1, Fig. 2, etc. Kymograph tracings should not be submitted with the original experimental legends which are likely to have been hastily or carelessly inscribed. Authors may find it worth while to employ a professional draftsman to make the drawings in proper form for reproduction.

Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretative statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript. Every table and every column should be supplied with an appropriate heading. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

NNR

PRODUCTS RECENTLY EVALUATED BY THE A. M. A. COUNCIL ON PHARMACY AND CHEMISTRY

BENZTROPINE METHANESULFONATE.—$C_{21}H_{25}NO.CH_4O_3S$.—Cogentin Methanesulfonate (Merck, S. & D.).—M.W. 403.52.—3-Diphenylmethoxytropane methanesulfonate.—Tropane benzohydryl ether methanesulfonate.—The structural formula of benztropine methanesulfonate may be represented as follows:



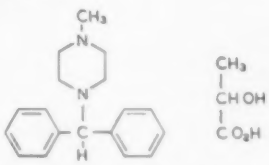
Actions and Uses.—Benztropine methanesulfonate, a parasympatholytic agent that comprises chemical features of both atropine and certain antihistaminic drugs, exhibits anticholinergic, antihistaminic, and local anesthetic properties. Its anticholinergic effect is useful in the symptomatic treatment of all forms of paralysis agitans. The therapeutic significance of its antihistaminic and local anesthetic actions in that disease or its overall usefulness for other conditions has not been established. The action of benztropine methanesulfonate is prolonged; its anticholinergic effect is approximately equivalent to that of atropine sulfate; its antihistaminic activity is similar to that of pyrilamine maleate. Benztropine may be associated with anticholinergic-like side-reactions as well as the characteristic sedative and other side-effects of the antihistaminic drugs. Benztropine methanesulfonate does not produce central stimulation.

In paralysis agitans, benztropine methanesulfonate is chiefly effective for the relief of tremor and rigidity. It also may ameliorate other troublesome symptoms such as sialorrhea, drooling, mask-like facies, oculogyric crises, and pain secondary to cramping and muscle spasm. It may be employed alone or in conjunction with certain other drugs of established value in paralysis agitans, depending upon its effectiveness in individual patients. Aged patients who do not tolerate cerebral-exciting agents may respond to benztropine methanesulfonate.

Therapeutic doses of benztropine methanesulfonate may cause such anticholinergic-like side-effects as dryness of the mouth, blurred vision, nausea, and nervousness. Unless these effects are severe or are accompanied by vomiting or difficulting in swallowing or speaking, they usually can be disregarded or the dosage reduced slightly. In excessive doses for a given patient, the drug may cause weakness and inability to move particular muscle groups, which require adjustment in dosage. Difficulty in urination may be encountered occasionally, and the possibility of producing or aggravating glaucoma resulting from the mydriatic effect of the drug should be borne in mind. In the event of severe reactions such as mental confusion, excitement, or vomiting, the drug should be withdrawn temporarily and then resumed at a lower dosage level. Patients exhibiting a sedative side-effect, which may be associated with the accompanying antihistaminic action, should not be permitted to operate machinery or appliances requiring alert attention.

Dosage.—Benztropine methanesulfonate is administered orally. The usual dosage in paralysis agitans is 1 to 2 mg. daily, with a range of 0.5 to 6 mg. Patients should be started on a single daily dose of 0.5 to 1 mg. with increments of 0.5 mg. every few days until the optimal tolerated dosage is reached. The increments can be added as a separate 0.5 mg. dose so that doses of 0.5 mg., or multiples thereof, one to four times daily may be administered. Younger patients with postencephalitic paralysis agitans may tolerate larger initial doses and, for maintenance therapy, may require 2 mg. two or three times daily. Older patients may be controlled by 1 mg. twice daily. Some patients may receive the greatest benefit with a single 2 mg. dose at bedtime, which is often sufficient to control symptoms for 24 hours; a single total daily dose administered at another time also may be effective for some patients. As with other therapy in paralysis agitans, the approach should be one of individual adjustment of dosage to obtain maximal symptomatic improvement with a minimum of undesired effects. When the drug is given to replace or supplement other drugs, the change should be gradual, with the dosage of the previous medication being reduced as the dosage for benztropine is increased.

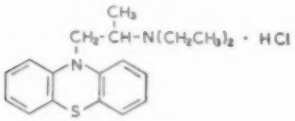
CYCLIZINE LACTATE.—Marezine Lactate (Burroughs Wellcome).—$C_{18}H_{22}N_2.C_3H_6O_3$.—M.W. 355.46.—1-diphenylmethyl-4-methylpiperazine lactate.—1-benzyhydryl-4-methylpiperazinel actate.—The structural formula of cyclizine lactate may be represented as follows:



Actions and Uses.—Cyclizine lactate has the same systemic actions as the hydrochloride salt of the drug. (See monograph on cyclizine hydrochloride.) The lactate is suitable for intramuscular injection when the oral route is not feasible for the control of motion sickness or vertigo caused by other vestibular disturbances. Its usefulness for the prevention or treatment of nausea and vomiting from other causes has not been established.

Dosage.—Cyclizine lactate is administered by intramuscular injection in a solution containing 50 mg./cc. The dosage is the same as for the orally administered hydrochloride. (See monograph on cyclizine hydrochloride.)

ETHOPROPAZINE HYDROCHLORIDE.—Parsidol (Warner - Chilcott).—$C_{19}H_{24}N_2S.HCl$.—M.W. 348.93.—10 - (2 - Diethylaminopropyl)phenothiazine hydrochloride.—The structural formula of ethopropazine hydrochloride may be represented as follows:



Actions and Uses.—Ethopropazine hydrochloride, a phenothiazine derivative, is a parasympatholytic agent that exhibits a ganglionic blocking action comparable to that of nicotine but that produces a sufficient depression of the central nervous system to block the convulsant activity characteristic of nicotine. Intense and prolonged mydriasis is produced by parenteral injection in experimental animals. The drug also antagonizes certain acetylcholine effects such as bradycardia, hyperperistalsis, and salivation but, unlike atropine, does not inhibit the hypotensive effects of acetylcholine. Except at high dosage levels, it has only a minor effect on blood pressure, and its adrenolytic activity is somewhat weak and variable. Small doses stimulate respiration, but larger doses depress respiration. The drug also exhibits some antihistamine activity.

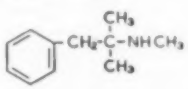
Ethopropazine hydrochloride is useful in the symptomatic treatment of parkinsonism, especially for controlling rigidity. It also exerts a favorable influence upon spasm, tremor, sialorrhea, and oculogyric crises and festination in that disease and may be employed in conjunction with other appropriate atropine-like drugs when these are indicated to supplement the action of ethopropazine, such as in refractory oculogyric crises. The role of its antihistaminic action in parkinsonism has not been established.

Ethopropazine hydrochloride commonly produces side-effects, but these are rarely severe. High dosage may cause temporary hypotension. With therapeutic doses drowsiness is the most frequent side-effect and dizziness also may occur. As dosage is increased, mouth dryness, blurring of vision, lassitude, sense of heaviness in the limbs, and, less commonly, epigastric discomfort also may appear. Because of its mydriatic action, ethopropazine theoretically should not be used in patients with glaucoma. Administration of the drug over long periods has not been followed by any blood dyscrasias or other signs of chronic toxicity.

Dosage.—Ethopropazine hydrochloride is administered orally. Optimal effective dosage is determined by individual response and may change with progression of the disease. Patients with symptoms of mild to moderate severity frequently respond to a daily dosage of 0.1 to 0.2 Gm., preferably administered in divided doses to provide a smooth, sustained effect of the drug. Severe symptoms may require gradual increase in the daily dosage to 0.5 or 0.6 Gm. Daily dosage as high as 1 Gm. has been employed without ill-effects, but it is seldom necessary to exceed a total of 0.7 Gm. daily. Therapy is usually initiated with a dose of 10 mg. four times daily. This is increased 10 mg. per dose every two or three days until a dose level of 50 mg. four times daily is reached at the end of the second week of therapy. Further increments may be added as necessary so as to reach an optimal level within three weeks of therapy. In some patients a fifth dose at bedtime may be desirable or the morning dose may be larger than the subsequent doses for the day. When used to supplement or supplant other medication, the drug is added to the existing regimen by administering initial doses and gradual increments as stated previously for two weeks; then it is continued while the original medication is gradually reduced. It is then stabilized at a lower level or is eventually discontinued. Complete transition usually requires a period of about six weeks; replacement therapy should not be substituted suddenly.

With the occurrence of distressing side-effects, lowering of dosage may become necessary, but those effects encountered during initial stages of therapy usually disappear with continued medication. Unless uncomfortable side-effects are unusually severe, it is rarely necessary to discontinue therapy.

MEPHENTERMINE.—Wyamine (Wyeth).—$C_{11}H_{17}N$.—M.W. 163.26.—*N*,α,α-Trimethylphenethylamine.—The structural formula for mephentermine may be represented as follows:



Actions and Uses.—Mephentermine is a sympathomimetic amine that shares the vasoconstrictor action common to such agents. It is sufficiently volatile for inhalation to produce vasoconstriction of the congested nasal mucous membrane, being about one-half as active in this respect as amphetamine. Mephentermine is less toxic than amphetamine when injected into experimental animals, and its pressor action resembles that of ephedrine rather than epinephrine; its cerebral stimulant action is much less than that of amphetamine and methamphetamine. It does not significantly relax smooth muscle. Local application of a 1% solution dilates the pupil. Mephentermine is used only by inhalation as a nasal decongestant to provide temporary relief in the treatment of acute or chronic rhinitis, allergic or nonallergic rhinitis, vasomotor rhinitis, and sinusitis.

Dosage.—Mephentermine is applied to the nasal mucosa by means of an inhaler containing a total of 250 mg. of the drug. Two inhalations through each nostril are recommended as a single dose. This provides a variable amount chiefly dependent upon the depth of inhalation. The contents of the inhaler is

The Massachusetts College of Pharmacy
Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the fields of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in this great medical and educational center. It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy.

For additional information, write to HOWARD C. NEWTON, Dean

WHO MAKES IT?

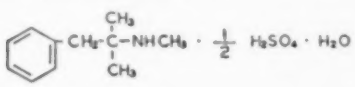
The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

2-Amino-1,3,5-triazine
Diphenylarsenious oxide
Glycine glycolate
Mucoitinsulfuric acid
N-Methyl anabasine
Naringenin glucoside-(2')
Naringenin glucoside-(5)
Octahydrophenazine
Phloroacetophenone glucoside
C_{21}-Phthianoic acid
C_{21}-Phthienoic acid
Phosphoryl choline
Phosphothreonine
Phosphotyrosine
Phosphohydroxyproline
Phosphohydroxylysine
3,3-bis(Ethylsulfonyl)pentane = tetronal
Tachysterol

N.N.R. • • • • • • • • • • • • • • • *from page IV*

sufficient to last about three months from the time of initial use.

MEPHENTERMINE SULFATE, U.S.P.—Wyamine Sulfate (Wyeth).—$C_{22}H_{34}N_2 \cdot H_2SO_4 \cdot 2H_2O$.—M.W. 460.64.—N,α,α-Trimethylphenethylamine sulfate.—The structural formula for mephentermine sulfate may be represented as follows:



Actions and Uses.—Mephentermine sulfate is a soluble salt of a sympathomimetic amine base and exhibits the vasoconstrictor action characteristic of such compounds. The solution is applied topically as a nasal decongestant in the treatment of vasomotor rhinitis, acute or chronic rhinitis, allergic or nonallergic rhinitis, and sinusitis. For other actions, see the monograph on mephentermine.

Dosage.—A 0.5% solution of the drug in isotonic sodium chloride solution is topically applied to the nasal mucosa by means of a dropper. The recommended schedule is two or three drops in each nostril every 4 hours or as required to temporarily relieve nasal congestion.

Handsome Durable Binders for Journals of the American Pharmaceutical Association

Combination Sets for Practical Pharmacy and Scientific Editions $5.50 a pair
For Single Editions $3.25 each

These binders open flat as a bound book. Made of durable imitation leather, they will preserve your journals permanently. Each cover holds 12 issues (one volume)....Do your own binding. Instructions easy to follow. Mail coupon for binders on 10 day trial.

.....**Mail Coupon Today**.....

SUCKERT LOOSE-LEAF COVER CO.
234 W. LARNED ST. DETROIT 26, MICH.

Send....binders for the Journal of the American Pharmaceutical Association.
....Practical Pharmacy Edition for years....Vols.....
....Scientific Edition for years.....Vols.....
....Combination for years.....Vols.....
Will remit $.....in 10 days or return binders.
Name.....
Address.....
City.....Zone.....State.....
Postage prepaid in U. S.—Foreign Extra

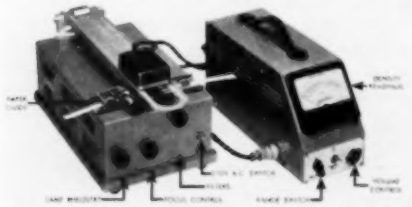
THE WELCH DENSICHRON®

TRANSMISSION DENSITOMETER

for PAPER CHROMATOGRAPHY

Provides a Simple and Remarkable Technique for Quantitative Micro-Analysis

Consists of Densichron, Probe, and Transmission Light Source



No. 3835B Patent No. 2424933

DENSICHRON WITH BLUE OR RED PROBE. This consists of the amplifier with logarithmic-scale meter, blue-sensitive probe, metal probe support, five different measuring apertures, a cone with 1/8-inch aperture, and a set of instructions. The amplifier operates on 115 volts, 60-cycle A.C., only, except on special order.

TRANSMISSION LIGHT SOURCE UNIT. This is an enclosed light source powered by a constant-voltage transformer and equipped with a hinged arm for supporting the Densichron probe. The plate or film to be observed is placed over the aperture and the probe is lowered into position for a reading. Suitable controls are provided. It operates on 115 volts, 60 cycles A.C.

No. 3835B
Complete
$425.00

W. M. WELCH SCIENTIFIC COMPANY
DIVISION OF W. M. WELCH MANUFACTURING COMPANY
ESTABLISHED 1880
1515 Sedgwick Street, Dept. AP, Chicago 10, Illinois, U.S.A.
Manufacturers of Scientific Instruments and Lab. Apparatus

FRL FOOD RESEARCH LABORATORIES, INC.
Founded 1922

RESEARCH
ANALYSES • CONSULTATION

Biological, Nutritional, Toxicological Studies for the Food, Drug and Allied Industries

48-14 33rd STREET
LONG ISLAND CITY 1, N. Y.

Bulletin "What's New in Food and Drug Research" available on letterhead request.

BOUND VOLUMES

Journal of the American Pharmaceutical Association

$11.00

Order from the Association

2215 Constitution Avenue, N. W.
Washington 7, D. C.

FINE REHEIS CHEMICALS

SINE CERA

SINE CERA—Translated literally, means—*without wax*. When used to describe an object of art in the days of early civilization in Rome, it signified perfection. It was a guarantee that there were no flaws or cracks that had to be filled with wax. This term is the basis for the present English word "sincere".

REHEIS—This label, when seen today by the pharmaceutical and cosmetic chemist, also signifies perfection and integrity. Reheis fine chemicals—SINE CERA—are accepted throughout the world as standards of the highest quality.

FINE REHEIS CHEMICALS

REHEIS COMPANY, INC.
Manufacturers of Fine Chemicals
BERKELEY HEIGHTS • NEW JERSEY

The ALCOLANS*

a series of self-emulsifying, emollient absorption bases whose active components are derived from pure lanolin.

ROBINSON WAGNER CO., INC.

Leaders in Lanolin Research

110 EAST 42nd ST. NEW YORK 17, N. Y.

Telephone: OXford 7-0277

Lustrous white, stable w/o emulsions are obtained by the simple addition of water. Emulsion consistency can be adjusted to almost any point desired between free-flowing liquid and solid paste state simply by varying the ratio of water to base.

The ALCOLANS are compatible with all ingredients used in pharmaceutical ointments and are *free from irritating and sensitizing properties.*

*Reg. U. S. Pat. Off.

For formulation data and further information request Product Bulletin 33.

USE SUPERIOR INGREDIENTS for SUPERIOR PRODUCTS

write ON YOUR BUSINESS LETTERHEAD TO

Amerchols are natural, non-ionic **OINTMENT BASES AND EMULSIFIERS** which contain ***Lanolin Cholesterols in their Most Active Form.***

These stable cholesterol and multisterol surfactants induce rapid drug release, promote optimum healing rates, and are safe for the most delicate tissues. We know of no case of an allergy due to an AMERCHOL.*

Modulan[T. M.] **is a chemically modified LANOLIN EMOLLIENT with unique new properties for use in OINTMENTS AND EMULSIONS. It imparts waxy, protective hydrophobic films; is oil soluble and compatible with O/W emulsions, soaps and shampoos. Clinical investigations indicate that MODULAN IS HYPO-ALLERGENIC.***

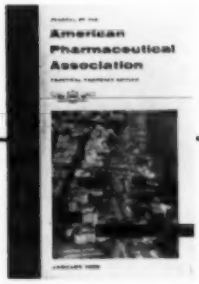
**References, technical data and suggested formulations are available from our research laboratories.*

AMERICAN CHOLESTEROL PRODUCTS

• INCORPORATED •

MILLTOWN • NEW JERSEY

AMERICAN CHOLESTEROL PRODUCTS, INC. AMERCHOL



A Preview of Advertising

In the April 1956 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ In its long-familiar, *looked-for* and *looked-at* position on the second cover, **Abbott Laboratories** delineates the "plus" factors of its dietary supplement, **Sur-Bex,** and elsewhere in a two-page spread, describes the efficacy of its antibiotic, **Erythrocin.**

▸ **The American Druggists' Insurance Company,** *For Druggists Only,* points up the role of insurance in providing *now* for possible future eventualities.

▸ In introducing **Clinitest,** a *new* urine-sugar test, **Ames** emphasizes its "utmost simplicity and convenience, complete specificity, extreme sensitivity, qualitative accuracy."

▸ A *new one-inch size* **Vaseline** gauze strip, manufactured by the Professional Products Division of the **Chesebrough Mfg. Co.,** is available through wholesalers or through the **Colgate-Palmolive Co.**

▸ **Lutrexin Tablets,** product of **Hynson, Westcott & Dunning,** is reported to have relieved, in many cases, "the entire symptom complex of dysmenorrhea."

▸ In a timely announcement, **Lederle Laboratories** delineates the efficacious effects of **Rhulicream**—indicated for the relief of pain and itching caused by poison ivy, insect bites and other minor skin irritations.

▸ "For better health during pregnancy," **Lilly** advocates **'Prenalac'** which "provides recommended daily vitamin and mineral allowances suggested by the Food and Nutrition Board of the National Research Council."

▸ In euphonious language, **J. B. Lippincott Company** reminds pharmacists that the new 25th edition of the **Dispensatory of the U.S.A.** is *indispensable* to . . . modern practice."

▸ The STORMOR bottle, fulfilling the promise implicit in its name, *is an exclusive* **Mallinckrodt** *package.*

▸ The **S. E. Massengill Company** says of **Salcort** that it is a "judicious" combination of salicylates and cortisone which results in "a much more favorable reaction in arthritis than with either alone."

▸ The **National Drug Company** offers the following "Products of Original Research": **Parenzyme, Hesper-C, Hesper-C Liquid, AVC, Protinal, Vi-Protinal, Resion** and **Resion P-M-S.**

▸ Enumerated by **Parke, Davis** in its **Combex** line are: *Combex Kapseals, Combex with Vitamin C Kapseals, Thera-Complex Kapseals, Combex Parenteral, Taka-Combex Kapseals and Taka-Combex Elixir.*

▸ **Bonamine,** offered by **Pfizer Laboratories,** is described as "the *only* motion-sickness preventive effective in a *single daily dose.*

▸ With customary perspicuity, the message of the **Philadelphia College of Pharmacy and Science** points up the sound future for which its schooling lays the foundation.

▸ Manufactured by **Pictorial Paper Package Corp.** is a *new* "Files-Well" Prescription File which holds *9,000* prescriptions.

▸ "Thoroughgoing relief for the patient with nonspecific rheumatism," is the description **Schering** tags on *new* **Sigmagen Tablets.**

▸ **Smith, Kline & French** adds still another affiant to the many testifying to the sales-effectiveness of its **Benzedrex** inhaler display carton.

▸ Virtually synonymous with the disability it remedies, is **Upjohn's Kaopectate,** recommended for diarrhea.

▸ Indicated "especially in the presence of cardiac and circulatory inadequacy" is **Peritrate** with *Aminophylline,* product of **Warner-Chilcott.**

▸ Advocated "whenever *reliable* analgesic action is needed" are **Winthrop Laboratories' A.P.C.** *with* **Demerol Tablets.**

▸ The **Wyeth** announcement emphasizes the prescription-potential inherent in **Equanil** and advocates the maintenance of adequate supplies to meet it.

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out April 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLV — *APRIL 1956* — NUMBER 4 — CONSECUTIVE NO. 8

Two Cyclic Analogs of Acetylcholine*

By F. W. SCHUELER

Cyclic compounds which imitate two spatial configurations of acetylcholine have been studied as to their muscarinic activities and hydrolyzability by acetylcholinesterase. The results lend support to the hypothesis that flexibility *per se* is a factor to be considered in the collation of chemical structure with pharmacologic activity.

MATHEMATICAL and experimental studies concerning diverse congeners of acetylcholine indicate that the collation of chemical structure with muscarinic or neuromuscular blocking activity is more reliable when drawn in terms of a statistical distribution of the steric factors involved than when single measurements serve as the basis for structural comparison (1, 2). Indeed, among the chainlike congeners of acetylcholine and decamethonium, flexibility *per se* (i.e., a wide distribution of molecular length) appears to be an important factor for high potency. Whether this latter arises out of a corresponding variability in the spacing of the primary receptor sites is, of course, unknown.

The present report emphasizes the above conception of flexibility as a factor pertinent to high muscarinic activity through the consideration of the pharmacologic activities of two cyclic analogs of acetylcholine (Fig. 1). These materials, I and II, are of special interest in so far as they are relatively rigid molecules analogous to the extreme configurations, III and IV, assumable by acetylcholine. The determination of the muscarinic potency of the analogs I and II, relative to acetylcholine, together with an assessment of their susceptibility as substrates of bovine erythrocyte cholinesterase were carried out.

* Received June 8, 1955, from the Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City.

This investigation was supported by a grant from Lakeside Laboratories, Inc.

EXPERIMENTAL

Materials.—The cyclic analog I, N,N-dimethyl-6-morpholonium iodide, was synthesized according to the scheme in Fig. 2. Five-tenths mole monochloroacetic acid and 1.0 *M* β-methylaminoethanol were mixed in a beaker cooled in an ice-water mixture. After the rapid exothermic reaction was over, the resulting thick syrup was allowed to stand overnight at room temperature. Then, following solution in an equal volume of water, 1.0 *M* sodium hydroxide (as a 40% solution) was added. The resulting mixture, after cooling, was extracted six times using for each extraction a volume of ether equal to the volume of the mixture. This treatment removes the excess 0.5 *M* of β-methylaminoethanol. The ether extract was discarded and the residue evaporated nearly to dryness on the water bath, cooled and finally treated with a sixfold volume of acetic anhydride. Following the initial reaction with acetic anhydride, during which some heat is evolved, the whole mixture was boiled for a few minutes and allowed to stand overnight, during which time a quantity of fine crystals was deposited (NaCl and NaAc). The crystals were removed by filtration and the filtrate subjected to distillation at atmospheric pressure (about 740 mm. of Hg). After a forerun of acetic anhydride and acetic acid a somewhat viscous oil was obtained over the temperature range 225–240°. This oil was redistilled, the portion boiling between 235–240° being collected. The oil, which is colorless when pure, has a slight ammoniacal odor. Quaternization of the oil was carried out by solution in an equal volume of ether followed by the addition of

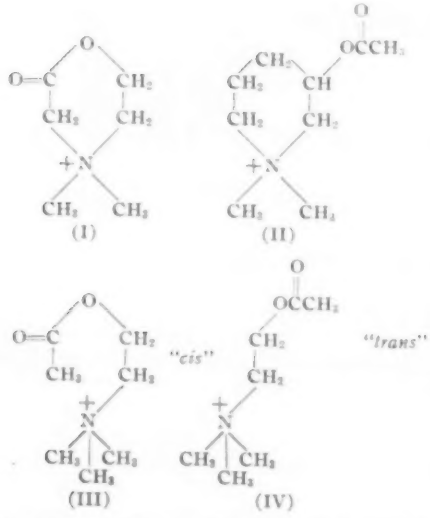


Fig. 1.—Comparison of the cyclic analogs with corresponding configurations of acetylcholine.

excess methyl iodide, the mixture being allowed to stand at least twenty-four hours. The white crystalline solid obtained was filtered off and washed repeatedly with peroxide-free dry ether and, finally, dried *in vacuo*. This substance obtained in an overall yield of about 30%, is freely soluble in water, and has a m. p. 239–240° (with decompn.).

Anal.—Calcd. for $C_6H_{12}O_2NI$: I, 49.21. Found: I, 49.4.

Determination of the acid (lactone) equivalent, by digestion of a 15-mg. sample in 10 cc. of 0.01 *N* sodium hydroxide followed by back titration with 0.01 *N* hydrochloric acid using phenolphthalein as an indicator, checked within 2% of the theoretical value.

A sample of analog II (Fig. 1), *N,N*-dimethyl-β-

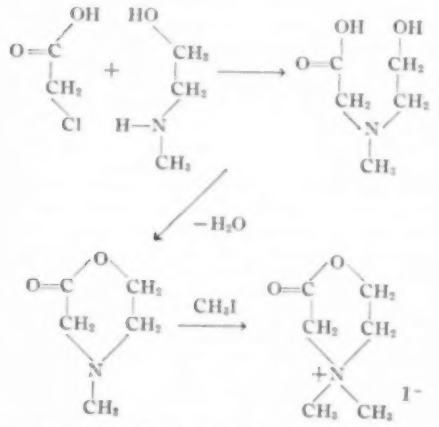


Fig. 2.—Scheme for the synthesis of cyclic analog I.

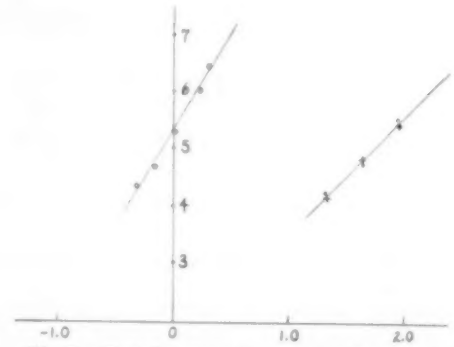


Fig. 3.—Dose-depressor response relationships on the dog arterial blood pressure: abscissa in $\log_{10}$ (dose μg./Kg.), ordinate in probits. +, ●, ○ denote, respectively, analogs I, II, and acetylcholine.

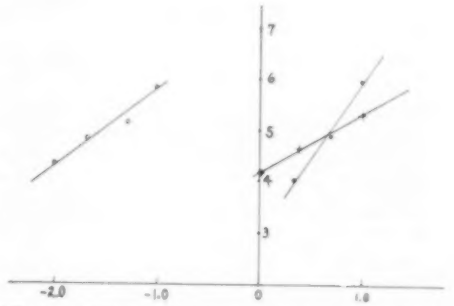


Fig. 4.—Dose-depressor response relationships (as in Fig. 3) following neostigmine bromide.

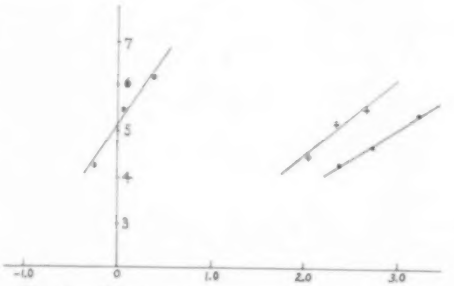


Fig. 5.—Dose-contraction response relationships using the isolated gut of the guinea pig. Abscissa in $\log_{10}$ (concentration in μg. %), ordinate in probits. Labeling as in Figs. 3 and 4.

acetoxypiperidinium bromide, was donated by Lakeside Laboratories, Inc.

Methods.—Using acetylcholine chloride as a standard, the products in question were evaluated in regard to their muscarinic depressor and gut stimulant activities following methods which have already been described in detail (3). Conventional manometric methods were used in the determination of their susceptibility as substrates for bovine

TABLE I.—RELATIVE MOLAR DEPRESSOR POTENCIES

	ED_{50} (μM/Kg.)		Activity Ratio[b]		
	Before Neostigmine[a]	After Neostigmine	Before Neostigmine	After Neostigmine	Potentiation Ratio[c]
Acetylcholine chloride	0.0035	0.000115	1	1	30.4
I	0.18	0.017	0.019	0.0068	10.1
II	0.23	0.018	0.015	0.0064	12.8

[a] The potentiating dose of neostigmine was 30 μg./Kg.

[b] Activity ratio = $\frac{ED_{50}\text{ acetylcholine}}{ED_{50}\text{ drug}}$.

[c] Potentiation ratio = $\frac{ED_{50}\text{ before neostigmine}}{ED_{50}\text{ after neostigmine}}$.

erythrocyte cholinesterase with acetyl-β-methylcholine as a standard (4).

All dose-response relationships were calculated and plotted in terms of probit-response *versus* $\log_{10}$ (dose in μg./Kg.). The curves so obtained were, in general, linear though often not parallel.

RESULTS AND DISCUSSION

Figures 3 and 4 represent the muscarinic depressor activity before and after neostigmine. Figure 5 represents the gut stimulant activity using the isolated ileum of the guinea pig and Fig. 6 the enzymic and spontaneous hydrolyzability. The depressor and gut stimulant responses caused by the analogs I and II were always effectively blocked by doses of atropine sulfate sufficient to inhibit the muscarinic responses to acetylcholine.

Table I exhibits the depressor effects of the analogs I and II relative to acetylcholine when the ED_{50} for each is calculated from the primary graphs on a molar basis. The drugs I and II were strikingly similar in depressor potency before and after the administration of neostigmine. Moreover, the degree of potentiation effected by neostigmine for both cyclic agents was of the same order of magnitude though neither agent was potentiated to as high a degree as acetylcholine.

As gut stimulants, analogs I and II, were also much less potent than acetylcholine though, in this test, analog I was somewhat more active than II (Fig. 5). Their susceptibilities to hydrolysis by cholinesterase are again similar (Fig. 6), but analog I was hydrolyzed very rapidly in a nonenzymatic fashion at the *p*H of the system (7.4) whereas analog II was relatively stable.

Analogs I and II represent, respectively, relatively rigid configurations assumable by the flexible molecule of acetylcholine. If analog I can be thought of as a material in which a "*cis*" type of configuration has been enforced through cyclization, analog II would represent a corresponding extended or "*trans*" form.

The *enforcement* of neither configuration appears to have significantly greater advantage over the other in enhancing muscarinic activity or susceptibility to cholinesterase hydrolyzability. Both situations, representing as they do a decrease in flexibility, confirm the results obtained previously (1) through the investigation of open chain analogs of II, that the flexibility of acetylcholine, as such, is an important attribute concerning the relationship between its structure and action. Whatever importance, therefore, is to be assigned to the nitrogen-ester group, intramolecular distance is best considered as a statistical distribution which may match an equivalent variability found in the receptors with which acetylcholine interacts.

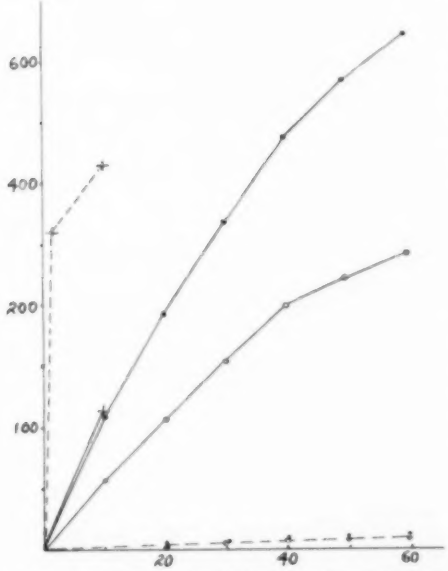


Fig. 6.—Enzymic (solid lines) and spontaneous hydrolysis (dash lines) abscissa in minutes, ordinate in total μL. of CO_2 evolved. +, ●, and ○ represent, respectively, analogs I, II, and acetyl-β-methylcholine.

SUMMARY

Two cyclic analogs of acetylcholine have been evaluated in regard to their muscarinic depressor and gut stimulant effects. The data lend support to the conjecture that flexibility of the acetylcholine molecule is an important attribute to be considered in collating structure with pharmacologic activity.

REFERENCES

(1) Schueler, F. W., *Arch. intern. pharmacodynamie*, **93**, 417(1953).
(2) Schueler, F. W., *ibid.*, **95**, 376(1953).
(3) Bass, W. B., Schueler, F. W., Featherstone, R. M., and Gross, E. G., *J. Pharmacol. Exptl. Therap.*, **100**, 465 (1950).
(4) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques in Tissue Metabolism," Burgse Publishing Co., Minneapolis, 1949, p. 28.

The Chemical Evaluation of *Rauwolfia serpentina* Preparations*

By JONAS CAROL, DANIEL BANES, JACOB WOLFF, and HERMAN O. FALLSCHEER

Methods are presented for the chemical identification and assay of *Rauwolfia serpentina* root and its preparations. The assay is based upon the determination of reserpine and rescinnamine. *Rauwolfia serpentina* is differentiated from other Rauwolfia species by the detection of characteristic alkaloids on paper chromatograms.

Rauwolfia serpentina root is notable for its relatively high concentration of certain characteristic indole alkaloids, especially trimethoxybenzoyl and trimethoxycinnamoyl alkamines (1). Reserpine and rescinnamine, the chief acyloxy alkaloids isolated from *Rauwolfia serpentina*, have been shown to possess both hypotensive and sedative properties similar to those of the crude drug (2). Although the root contains more than twenty alkaloids with various physiological activities (3), Wilkins has asserted that clinically reserpine and *Rauwolfia serpentina* root exhibit hypotensive and sedative properties which are practically identical (4). It seems feasible, therefore, to employ the reserpine–rescinnamine content as an index for evaluating the sedative and hypotensive potency of the powdered whole root or its preparations.

In the proposed assay procedure, a fraction containing reserpine and rescinnamine, together with other weakly basic alkaloids, is separated from stronger bases by liquid–liquid partition chromatography (5). The reserpine–rescinnamine content is then calculated from the quantities of trimethoxybenzoic and trimethoxycinnamic acids recovered after saponification of the mixed collutes.

The proposed chromatographic identification tests serve to distinguish *Rauwolfia serpentina* from other Rauwolfia species. One of these tests (Procedure A) has been described previously (5). Procedure B permits the separation of alkaloids with relatively high R_f values. Paper chromatograms prepared from *Rauwolfia serpentina* extracts show fluorescing spots corresponding to reserpine, rescinnamine, reserpinine, ajmalicine, ajmaline, and serpentine, *inter alia*. No other species treated in an identical manner exhibits all of these fluorescing spots (Fig. 1 and 2). It is especially noteworthy that significant quantities of rescinnamine occur only in *Rauwolfia serpentina* and *Rauwolfia vomitoria*, and the latter lacks both ajmalicine and reserpinine.

* Received February 2, 1956, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

EXPERIMENTAL

Proposed Assay for Reserpine and Rescinnamine

Adsorbant.—Boil a mixture of 150 Gm. of Celite® 545 and a liter of diluted hydrochloric acid for 10 minutes. Cool, filter by suction, wash thoroughly with water until the washings are neutral to methyl orange, and dry overnight at 100°. Ignite at 500° for two hours.

Immobile Solvent.—Dissolve 10.50 Gm. of citric acid in water, add 10.0 ml. of 1.00 *N* sodium hydroxide solution, dilute to 500 ml., and mix thoroughly. Mix 50.0 ml. of the citrate buffer solution with 20.0 ml. of alcohol, and cool to room temperature.

Eluent.—Mix exactly 200 ml. of isooctane, 100 ml. of chloroform, 100 ml. of water, and 40 ml. of alcohol. Shake for 20 minutes, discard the lower aqueous phase and filter rapidly through paper.

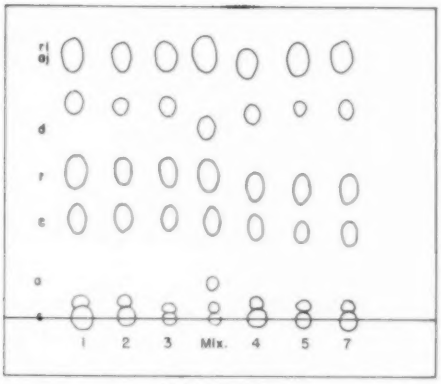
Chromatographic Tube.—A 400-mm. length of glass tubing (i.d. 22–23 mm.) fused to a piece of outlet tubing.

Chromatographic Column.—Place a small pledget of cotton in the bottom of the tube. Thoroughly mix 1.5 Gm. of adsorbant, 1 ml. of 2% sodium bicarbonate solution, and 0.4 ml. of alcohol. Add to the tube and tamp down. Add in several portions, tamping after each addition, a uniform mixture of 10 Gm. of adsorbant and 10 ml. of alcoholic citric acid solution. Use about 0.5 Gm. of adsorbant to rinse out the beaker. The alcoholic citric acid layer should be about 11 cm. high.

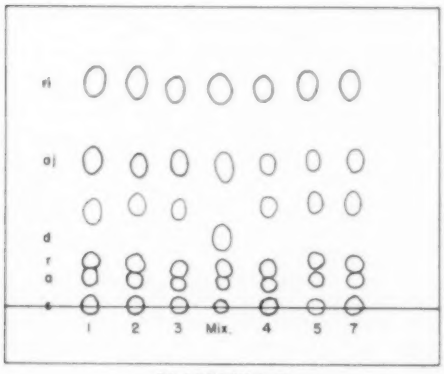
Sample Preparation.—Accurately weigh 2.5–3 Gm. of ground root, or its equivalent in tablets or extracts, and mix with 20.0 ml. of alcohol. Heat at 55–65° for 30 minutes in a tightly-stoppered flask. Cool, add 30.0 ml. of chloroform, and shake for 30 minutes. Filter rapidly through paper.

Procedure.—Mix a 10.0-ml. aliquot of the sample filtrate with 12 ml. of isooctane. Stir in 15 Gm. of adsorbant previously mixed with 10 ml. of 2% sodium bicarbonate. Mix until uniform. Transfer quantitatively to the chromatographic tube in several portions, tamping after each addition. Rinse the container with a Gm. of adsorbant, and add to the column.

Percolate eluent through the column at a rate of 2–4 ml. per minute. Discard a forerun of $(t + 10)$ ml., where t is the temperature in degrees centigrade. Collect the next 110 ml. of eluate, add 10 ml. of

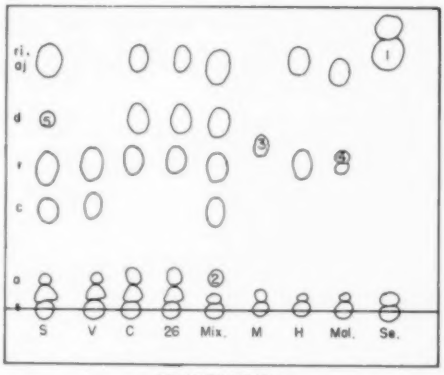


PROCEDURE A

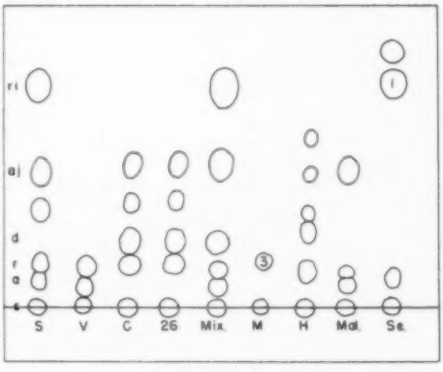


PROCEDURE B

Fig. 1.—Paper chromatograms of *R. serpentina*. Sample numbers correspond to those in Table II. Mix = Mixture of seven alkaloids: ri = reserpinine; aj = ajmalicine; d = deserpidine; r = reserpine; c = rescinnamine; a = ajmaline; s = serpentine.



PROCEDURE A



PROCEDURE B

Fig. 2.—Paper chromatograms of various Rauwolfia species. S = *Rauwolfia serpentina;* V = *Rauwolfia vomitoria;* C = *Rauwolfia canescens;* M = *Rauwolfia micrantha;* H = *Rauwolfia hirsuta;* Mal. = *Rauwolfia malabar;* Se = *Rauwolfia sellowii.* For Sample 26, see references in the *Discussion* and in Table II. Fluorescence of numbered spots: 1 = Bright orange; 2, 3 = deep blue (weak); 4 = orange (weak); 5 = very weak, fluoresces like deserpidine.

alcohol, and evaporate to dryness *in vacuo* at a temperature of 50–60°.

Dissolve the residue in 15 ml. of hot alcohol, add 5 ml. of sodium hydroxide T.S., cover with a watch glass and heat on a steam bath for 20 minutes. Cool, and transfer the hydrolysate to a separator with several small portions of water. Make just acid with diluted sulfuric acid, then add an excess of 5% sodium bicarbonate solution. Mix carefully and extract with three 20-ml. portions of chloroform. Wash the chloroform layers in succession with 10 ml. of 0.5% sodium bicarbonate solution and discard.

Combine the aqueous solutions and render distinctly acid with diluted sulfuric acid solution. *Mix carefully.* Extract with one 10-ml. and three 4-ml. portions of chloroform. Wash the chloroform extracts in succession with 10 ml. of water. Filter the extracts through a pledget of purified cotton washed with chloroform, dilute to exactly 25 ml. with chloroform, and mix.

Prepare a solution of trimethoxybenzoic acid in chloroform containing 10 μg. per ml., and a similar solution of trimethoxycinnamic acid. Measure the absorbances of these solutions, and of the sample solution, at 270 and 300 mμ in a suitable spectrophotometer relative to a chloroform blank. Let A_{270} and A_{300} equal the absorbances of the sample solution, B_{270} and B_{300} those of the trimethoxybenzoic acid solution, and C_{270} and C_{300} those of the trimethoxycinnamic acid solution. The weight of reserpine in the charge analyzed, in milligrams, is given by the formula:

$$3.59\,\frac{(A_{270} \times C_{300} - A_{300} \times C_{270})}{B_{270} \times C_{300} - B_{300} \times C_{270}}$$

and the weight of rescinnamine in the charge analyzed is given by the formula:

$$3.34\,\frac{(A_{300} \times B_{270} - A_{270} \times B_{300})}{B_{270} \times C_{300} - B_{300} \times C_{270}}$$

Identification Tests

Formamide.—Shake formamide with anhydrous potassium carbonate. Filter and distill *in vacuo* using all glass apparatus (b. p. 101° at 12 mm. Hg). Store in a desiccator over sulfuric acid until free of ammonia.

Trichloroacetic Acid Spray Reagent.—Dissolve 5 Gm. of trichloroacetic acid and 25 mg. of sodium nitroprusside in 100 ml. of methanol (6).

Immobile Solvent.—Dilute 30 ml. of formamide to 100 ml. with acetone.

Mobile Solvent (A).—Shake 60 ml. of *n*-heptane, 40 ml. of carbon tetrachloride, and 2 ml. of formamide for 5 minutes. Discard the lower layer, filter through paper and mix with 2.5 ml. of *t*-butyl alcohol.

Mobile Solvent (B).—Shake 40 ml. of benzene, 60 ml. of isooctane and 2 ml. of formamide for five minutes. Discard the lower layer and filter.

Sample Preparation.—Reduce 10 Gm. of root to a fine powder. Warm a 1-Gm. portion with 5 ml. of alcohol at 55–65° for 30 minutes. Cool and filter. Prepare an alcoholic reference solution containing 200 μg. of reserpine per ml.

Paper Chromatography

Procedure A.—Proceed as directed in (5), using an atmosphere of ammonia in a chamber suitable for ascending chromatography, and mobile solvent A instead of the benzene-isooctane-cyclohexanol solvent. After drying the paper at 90° in a current of air, spray lightly with trichloroacetic acid reagent and dry again at 90° for 10 minutes. Examine the chromatogram under an ultraviolet lamp, and note the position of the fluorescent spots.

Procedure B.—Chromatograph as in procedure A, but use mobile solvent B and no atmosphere of ammonia.

DISCUSSION

The proposed method of assay was applied to a specimen of pure crystalline reserpine and to a sample of rescinnamine apparently containing about 7% reserpine and 5% other contaminants. Analytical data for these controls, as well as recoveries of alkaloids added to root samples are listed in Table I. Although variations were encountered in the determination of individual alkaloids, the overall recovery as total apparent reserpine plus rescinnamine ranged from 94 to 99%, probably due to compensating interferences. Similarly, data obtained on replicate analyses of powdered roots and root extracts showed better agreement for total reserpine plus rescinnamine than for the individual alkaloids (Table II). It is clear that the total reserpine plus rescinnamine content is the most reliable criterion available by this procedure, as should be expected from the interdependence of the values yielded by two-component spectrophotometry.

Data obtained by application of the proposed assay procedure to roots, tablets, and extracts are listed in Table II. The agreement between analysts, and between replicate analyses demonstrates satisfactory reproducibility. Assuming a normal range of 150–250 mg. of reserpine plus rescinnamine per 100 Gm. of authentic *Rauwolfia serpentina* root, all of the tablet samples assayed except Sample 20 were found to contain the declared quantity of crude drug. In the case of Sample 27, the method failed because one of the coating dyes persisted throughout the procedure. In Sample 28, an emulsifying agent interfered with the chromatographic development. Extract 26, allegedly derived from *Rauwolfia serpentina*, was proved spurious by paper chromatography. Its chromatogram most nearly resembled that given by *Rauwolfia canescens* (Fig. 2). Extract 24 contained smaller concentrations of alkaloids than anticipated from the label declaration, assuming 2% total alkaloids for *Rauwolfia serpentina* root.

Examination of the chromatographic columns under ultraviolet light after removal of the reserpine-rescinnamine fraction revealed the presence of several fluorescing bands near the top of the alcoholic citrate trap layer. These bands include other trimethoxybenzoyl and trimethoxycinnamoyl alkamines, whose nature has not been established. The paper chromatograms exhibited several fluorescing spots not correlated with the seven known alkaloids tested. Several of these spots are indicated numerically in Fig. 2, together with their characteristic fluorescent colors. All of the known alkaloids fluoresced blue-green, except serpentine (bright blue) and deserpidine (yellow-green).

TABLE I.—ANALYSIS OF CONTROL SAMPLES

Control	Description	Reserpine, mg. Theoretical	Reserpine, mg. Recovered	Rescinnamine, mg. Theoretical	Rescinnamine, mg. Recovered	Total Recovered, %
C-1	2.0 mg. Reserpine	2.00	1.91	0.00	0.00	95.5
	2.0 mg. Reserpine	2.00	1.94	0.00	0.00	97.0
	2.0 mg. Reserpine	2.00	1.90	0.00	0.03	96.5
C-2	1.136 mg. Rescinnamine	0.08	0.059	1.00	0.98	96.2
	1.136 mg. Rescinnamine	0.08	0.084	1.00	0.99	99.4
	1.136 mg. Rescinnamine	0.08	0.080	1.00	0.99	99.1
C-3	1.0 mg. Reserpine + 0.28 mg. Rescinnamine	1.02	0.99	0.25	0.23	96.1
C-4	Sample 6[a] + 2.50 mg. Reserpine	6.42	6.26	1.96	1.89	97.3
C-5	Sample 6 + 1.40 mg. Rescinnamine	3.96	4.04	3.16	3.04	99.4
C-6	Sample 1[a] + 2.48 mg. Reserpine + 1.94 mg. Rescinnamine	6.13	5.75	3.00	2.89	94.6
C-7	Sample 6 + 2.50 mg. Reserpine + 1.40 mg. Rescinnamine	6.60	6.05	3.23	3.16	93.7

[a] Sample numbers correspond to those listed in Table II.

TABLE II.—ANALYSIS OF RAUWOLFIA SERPENTINA PREPARATIONS

Sample	Description	Alkaloids, μg./100 mg. or μg./tablet[a]					
		Reserpine		Rescinnamine		Total	
		Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
1a	Whole root		133		49		182
b		134		52		186	
2a	Whole root		166		72		238
b		164		81		245	
3a	Whole root	115		51		166	
b			113		47		160
4a	Whole root	164, 166		72, 74		236, 240	
b			160		76		236
5a	Whole root	136	141	70	69	206	210
b		142, 138		71, 70		213, 208	
6a	Powdered root	140	139	68	63	208	202
b		142		70		212	
7	Powdered root	106		55		161	
8a	Whole root	119		70		189	
b		119		61		180	
9	Whole root (Thailand)	120	116	84	86	204	202
10a	Powdered root	113	109	57	53	170	162
b		113	112	54	57	167	169
11	Tablets, 100 mg.	101		53		154	
12	Tablets, 100 mg.	122	118	60	57	182	175
13	Tablets, 4 mg. total alkaloids	216	219	107	97	323	316
14	Tablets, 50 mg.	64	62	31	29	95	91
15	Tablets, 100 mg.	133	132	67	65	200	197
16	Tablets, 100 mg.	167		62		229	
17a	Tablets, 100 mg.	121	126	60	62	181	188
b		125		63		188	
18	Tablets, 100 mg.	126		45		171	
19	Tablets, 50 mg.	76	76	40	42	116	118
20	Tablets, 50 mg.	53	54	18	19	71	73
21	Tablets, 100 mg.		125		42		167
22	Extract, 25–35% reserpine	33,000		17,100		50,100	
23	Extract, 25–35% reserpine	32,500		17,800		50,300	
24a	Extract, 10% total alkaloids	364		231		595	
b		349		250		599	
25a	Extract, unknown strength	3,330		1,400		4,730	
b		3,420		1,340		4,760	
26	Extract, unknown strength	Not Serpentina					
27	Tablets, 50 mg.	Method failed					
28	Tablets, 50 mg.	Method failed					

[a] Data on horizontal lines represent values obtained on aliquots of the same sample solution. Letters (a) and (b) refer to different sub-samples.

SUMMARY

Methods have been proposed for the chemical identification and assay of *Rauwolfia serpentina* root and its preparations, based upon the presence of characteristic indole alkaloids. These methods have been applied successfully to many commercial products containing ground root or root extracts.

REFERENCES

(1) Banes, D., and Carol, J., *J. Assoc. Offic. Agr. Chemists*, **38**, 866(1955).

(2) Bein, H. J., *Experentia*, **9**, 107(1953); Slater, I. H., Rathbon, R. C., Henderson, F. G., and Neuss, N., *Proc. Soc. Exptl. Biol. Med.*, **88**, 293(1955); Cronheim, G., Brown, W., Cawthorne, J., Toekes, M. I., and Ungari, J., *ibid.*, **86**, 120(1954).

(3) Phillips, D. D., and Chadha, M. S., THIS JOURNAL, **44**, 553(1955).

(4) Wilkins, R. W., *Ann. N. Y. Acad. Sci.*, **59**, 36(1954).

(5) Banes, D., Carol, J., and Wolff, J., THIS JOURNAL, **44**, 641(1955).

(6) Glazko, A. J., Dill, W. A., Wolf, L. M., and Kazencho, A., *Federation Proc.*, **14**, 58(1955).

The Effect of Various Preservatives on Microorganisms Isolated from Deteriorated Syrups*

By JEROME SCHIMMEL† and WILLIAM J. HUSA

The activity of eight chemical preservatives and one hundred and twenty-six different proportions of twenty-six different mixtures of two, three, four, and five esters of *p*-hydroxybenzoic acid was tested against four microorganisms isolated from pharmaceutical syrups. Storage tests were conducted in syrup, U. S. P., and diluted syrup (42.5% sucrose w/v) under two different storage conditions for 60 days. *o*-Phenylphenol was most effective against the mold fungi and the yeast and benzyl *p*-hydroxybenzoate was the most effective against the bacterium. The following preservatives appeared to be the most effective for syrup, U. S. P.: *o*-phenylphenol at 1:15,000; propyl *p*-hydroxybenzoate at 1:5000; methyl, ethyl, propyl, butyl, and benzyl esters of *p*-hydroxybenzoic acid in the proportion 15:15:15:15:40 w/w at 1:7500. Mixtures of the *p*-hydroxybenzoates exhibited a potentizing effect in both the antimicrobial tests and storage studies. Antimicrobial studies using the FD&C colors showed that FD&C Red No. 3 was effective against the bacterium at 1:20,000, and FD&C Orange No. 1 was effective against the bacterium at 1:4700 and against the yeast at 1:3000.

DETERIORATION of syrups may be due to the effect of the atmosphere, the effect of heat and light, internal reactions due to inherent instability, action of the components of the preparation, and action of microorganisms (1).

Of the various methods which have been employed to prevent the microbial deterioration of syrups the use of chemical preservatives gives indications of affording the best solution to the problem. The selection of a chemical preservative is not an easy task for the microorganisms which have been isolated from deteriorated syrups comprise literally all the major genera of microorganisms (2).

The present study was undertaken to investigate the action of various preservatives against various microorganisms isolated from deteriorated syrups. Particular emphasis was placed on mixtures of esters of *p*-hydroxybenzoic acid (*p*-hydroxybenzoates).

* Received July 22, 1955, from the College of Pharmacy, University of Florida, Gainesville.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.
This paper is based on a dissertation presented to the Graduate Council of the University of Florida by Jerome Schimmel, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Present Address: Hart Drug Corporation, Miami, Florida.

Lord and Husa (3) evaluated more than two hundred substances as antimolding agents for syrups employing an accelerated test method. A subsequent study by Littlejohn and Husa (4) showed that there was a definite potentizing action when mixtures of *p*-hydroxybenzoates were used as preservatives for syrups. Aalto, Firman, and Rigler (5) cite additional references to this phenomenon.

EXPERIMENTAL

Antimicrobial Studies.—Before storage experiments could be conducted with syrups containing various concentrations of preservatives, it was necessary to determine which microorganisms occurred most frequently as contaminants in syrups and to ascertain the minimal concentration of preservative that would prevent their growth on agar media. These data would then serve as a guide for the concentrations of preservative to add in subsequent storage experiments.

Isolation and Selection of Microorganisms.—The microorganisms used in this investigation were the most frequently occurring contaminants isolated by plate-dilution technique from twenty-five pharmaceutical syrups. These microorganisms were *Aspergillus niger*, *Penicillium glaucum*, *Proteus vulgaris*, and a yeast of the *Zygosaccharomyces* genus.

Preservatives Tested.—The activity of benzoic acid, sorbic acid, *o*-phenylphenol, and the methyl, ethyl, propyl, butyl, and benzyl esters of *p*-hydroxybenzoic acid was tested against the four isolates.

Procedure.—The media were measured into four-ounce prescription bottles, loosely capped, and autoclaved at 121° for fifteen minutes. Mycophil Agar (B. B. L.) was employed in tests with the *Aspergillus*, *Penicillium*, and the yeast; Nutrient Agar (Difco) was employed in the bacterial tests. The preservatives were dissolved in alcohol at such concentrations that, when 1.0 cc. was added to 50 cc. of medium, the desired concentration was obtained. Increments of 1:250 were generally used. All additions were made to sterile media which were liquefied and held at 45°.

A spore suspension of the mold was made by pipetting 20 cc. of sterile distilled water to a mature slant, scraping the surface thoroughly, decanting, and filtering. One-tenth cubic centimeter of the spore suspension and approximately 25 cc. of the medium were added to the Petri dish which was agitated to insure uniform dispersion of the inoculum. When the medium had solidified, the dish was incubated at 28–30° for seven days and the growth was read and recorded as either positive or negative. Blanks of uninoculated medium and

culture control containing 1.0 cc. of alcohol were run.

Tests on the yeast and bacterium were conducted in essentially the same manner as described for the molds with the exception that the medium was poured into the dish and allowed to solidify before inoculation. In this case, a loopful of a suspension prepared from 10 cc. of sterile distilled water added to a 24-hour slant of the organism was drawn across one sector of the dish. Readings were made after 24 hours incubation at 30–32° and recorded as either positive or negative.

All tests were carried out in duplicate at three different times.

General Storage Tests.—The preservatives used in the antimicrobial studies were tested in syrup, U. S. P., and diluted syrup (42.5 per cent sucrose w/v) maintained under two different storage conditions: (*a*) on an east window sill which allowed the direct sun rays to strike the bottle for several hours a day, and (*b*) in a refrigerator which had an average temperature of 5°. Samples of syrup, U. S. P., were prepared by the hot process; samples of diluted syrup were prepared by adding distilled water to syrup, U. S. P., so that the resulting sucrose concentration was 42.5 per cent w/v. All storage tests were conducted in duplicate for a period of 60 days.

The preservative was dissolved in alcohol at such concentration that, when 10 cc. was added to 200 cc. of syrup, the desired concentration was obtained. Addition of the preservative to syrup was made at 70°. The resulting solution was divided into four 50-cc. portions, placed in two-ounce prescription bottles, and loosely capped. When the syrups had cooled to room temperature, the caps were securely tightened and the bottles gently agitated to incorporate the condensate formed in the unfilled portion of the bottle. The results of this experiment may be found in Table II.

Stored Inoculated Syrups.—Samples of syrup, U. S. P., and diluted syrup containing the same preservatives at the concentrations used in the general storage tests were inoculated with a suspension of *Aspergillus*. The inoculated syrups, which contained about 3,000 viable spores per cc., were stored under the same conditions for 60 days. In all but one case, *o*-phenylphenol at a concentration of 1:10,000, the growth of the *Aspergillus* was not inhibited by the preservatives in either of the syrups under either storage condition. These results may be due to the relatively high initial viable spore count.

Potentizing Action of Mixtures of *p*-Hydroxybenzoates.—Several investigators (4, 5) have suggested the use of mixtures of the *p*-hydroxybenzoates and have demonstrated an increase in activity when mixtures were employed. Since many of these reports are lacking in details, a comprehensive study of this potentizing action was undertaken. Throughout this study, the term "potentizing action" denotes the increased effect which occurs when a mixture of two or more preservatives has a greater apparent preservative action than the sum of the effects of the preservatives when used alone.

TABLE II.—MINIMAL CONCENTRATION OF PRESERVATIVE REQUIRED TO PRESERVE SYRUPS UNDER DIFFERENT STORAGE CONDITIONS FOR SIXTY DAYS

Preservative	Condition[a]	Syrup, U. S. P.	Syrup (42.5% Sucrose w/v)
Benzoic acid	1	1:750	1:1500
	2	<1:1000	<1:2000
Sorbic acid	1	<1:1000	1:1500
	2	<1:1000	<1:2500
o-Phenylphenol	1	1:15,000	1:35,000
	2	<1:50,000	<1:50,000
Esters of *p*-hydroxybenzoic acid:			
Methyl	1	1:1000	1:1500
	2	<1:1250	1:1500
Ethyl	1	1:2000	1:2500
	2	<1:2500	<1:3000
Propyl	1	1:5000	<1:6000
	2	<1:5500	<1:6000
Butyl	1	1:4500	<1:8000
	2	<1:10,000	<1:8000
Benzyl	1	1:11,000	<1:20,000
	2	<1:20,000	<1:20,000

[a] Conditions: 1. Storage in direct sun rays. 2. Storage in refrigerator, 5°.

***p*-Hydroxybenzoate Mixtures Tested.**—One hundred and twenty-six different proportions of twenty-six different mixtures of two, three, four, and five esters were tested. These proportions were as follows: 5 proportions of each of 10 different mixtures of 2 of the 5 esters, 4 proportions of each of 10 different mixtures of 3 of the 5 esters, 5 proportions of each of 5 different mixtures of 4 of the 5 esters, and 11 proportions of 1 mixture of the 5 esters.

The calculation of potentizing action is the same as that employed by Littlejohn and Husa (4).

Antimicrobial Studies.—These tests were conducted in the same manner as previously described. The initial tests were made with the *Aspergillus* sp., since this organism was found to be the most frequent contaminant.

Results indicated that the activity of mixtures of

TABLE I.—ANTIMICROBIAL ACTIVITY OF PRESERVATIVES TESTED

Preservative	Ratio Required for Inhibition			
	Aspergillus	*Penicillium*	Bacterium	Yeast
Benzoic Acid	1:750	1:1250	1:3000	1:1000
Sorbic Acid	1:750	1:1000	1:3000	1:1000
o-Phenylphenol	1:21,000	1:30,000	1:8000	1:17,500
Esters of *p*-Hydroxybenzoic Acid:				
Methyl	1:1500	1:2000	1:750	1:1000
Ethyl	1:2500	1:4000	1:1500	1:2750
Propyl	1:4500	1:8000	1:3500	1:6500
Butyl	1:6500	1:10,500	1:12,000	1:10,500
Benzyl	1:9000	1:15,000	1:22,500	1:17,000

p-hydroxybenzoates was additive and in some cases potentizing. Nineteen different proportions of ten different mixtures showed a potentiation of 10 per cent or more, with the greatest potentiation, 35.4 per cent, exhibited by a mixture of the methyl, ethyl, propyl, and butyl esters (70:10:10:10 w/w) at a concentration of 1:3250.

The activity of these nineteen proportions was tested against the *Penicillium*, the bacterium, and the yeast. These tests confirmed that the activity of the *p*-hydroxybenzoates was additive and in some cases potentizing. A mixture of the methyl, ethyl, propyl, and butyl esters (70:10:10:10 w/w) showed the greatest potentiation against the *Penicillium* and the yeast (30.2% at a concentration 1:4750 on the former and 58.9% at a concentration 1:4250 on the latter). A mixture of the methyl, ethyl, and butyl esters (10:10:80 w/w) showed the greatest potentiation against the bacterium, 57.9 per cent at a concentration 1:2250. The first mixture was the only one that showed a potentiation of any considerable extent (30% or more) against all the microorganisms (refer to Table III).

U. S. P., stored in direct sun rays for 60 days were determined. Calculation of potentiation was made as previously described and the calculated values were based on the minimal effective concentrations of the respective esters employed. Thirty-nine of the 126 proportions tested showed a potentizing effect. Three proportions exhibited a potentiation of over 60%. These were methyl, ethyl, propyl, and butyl (70:10:10:10 w/w) 62.2% at concentration 1:3000, ethyl, propyl, butyl, and benzyl (10:70:10:10 w/w) 100% at concentration 1:4500; and methyl, propyl, butyl, and benzyl (10:70:10:10 w/w) 62.8% at concentration 1:3500.

Of the proportions which exhibited potentiation, a mixture of the methyl, ethyl, propyl, butyl, and benzyl esters (15:15:15:15:40 w/w) had the lowest minimal effective concentration, 1:7500, and a potentiation of 20.5%.

Antimicrobial Tests on the FD&C Colors.—Quite often, in the manufacture of flavored syrups for use in the beverage industry and at soda fountains, the FD&C colors are employed. These substances were tested to ascertain whether they possessed

TABLE III.—PER CENT INCREASE IN POWER OF MIXTURES OF PARAHYDROXYBENZOATES TO INHIBIT THE GROWTH OF MICROORGANISMS ISOLATED FROM SYRUP

Esters	Proportions	Per Cent Increase			
		Aspergillus	*Penicillium*	Bacterium	Yeast
	Group I.—Mixtures of Two Esters				
Methyl-propyl	35–65	15.9	0.7	8.3	3.8
Ethyl-butyl	90–10	29.3	7.5	7.8	54.5
	65–35	21.8	7.6	11.1	−3.9
	50–50	16.7	6.9	7.4	1.9
	35–65	17.6	12.5	2.1	3.8
	10–90	18.9	9.2	0.5	0.2
Butyl-benzyl	90–10	11.1	5.4	7.3	39.0
	Group II.—Mixtures of Three Esters				
Methyl-ethyl-propyl	80–10–10	18.4	15.7	−9.1	1.5
Methyl-ethyl-butyl	10–10–80	11.8	8.3	57.9	−1.4
Methyl-propyl-butyl	1/3 of each	25.9	6.1	1.5	0
	80–10–10	19.6	8.7	−6.8	−10.0
Propyl-butyl-benzyl	10–80–10	10.7	3.0	7.3	34.9
	10–10–80	14.5	1.1	35.5	−2.0
	Group III.—Mixtures of Four Esters				
Methyl-ethyl-propyl-butyl	70–10–10–10	35.4	30.2	46.1	58.9
	10–10–10–70	15.7	20.0	5.8	−1.5
Ethyl-propyl-butyl-benzyl	10–70–10–10	16.2	−0.6	−0.8	0.1
	10–10–70–10	17.9	−0.5	3.1	0.3
	Group IV.—Mixtures of Five Esters				
Methyl-ethyl-propyl-butyl-benzyl	10–10–10–60–10	19.5	8.7	4.7	−3.1
	15–15–15–40–15	10.1	2.3	−3.4	51.6

Storage Tests Using *p*-Hydroxybenzoate Mixtures.—In an attempt to reduce the quantity of ester required, storage tests were conducted with the previously tested mixtures and proportions of the *p*-hydroxybenzoates to determine the minimal effective concentrations required to preserve the syrups for 60 days. These tests were carried out in the same manner as previously described and were run in duplicate.

Due to insufficient range of dilutions, minimal concentrations were not determined for syrup, U. S. P., stored in the refrigerator or for diluted syrup under either of the storage conditions. The minimal concentrations required to preserve syrup, any previously unsuspected preservative effects. The media, equipment, and procedure for testing were the same as previously described.

Preliminary experiments indicated that many of the colors were microbially contaminated. All subsequent experiments were conducted with aqueous solutions of the colors which had been sterilized by autoclaving for 15 minutes at 15 pounds pressure (121°). There seemed to be no apparent color change due to sterilization.

None of the FD&C colors tested showed inhibition of the *Aspergillus* and *Penicillium* at a 1:500 concentration. At a concentration of 1:500 FD&C Orange No. 1 showed inhibition of both the

bacterium and the yeast, and FD&C Red No. 3 showed inhibition of the bacterium. Further tests showed that the former inhibited the bacterium at a minimal concentration of 1:4700, and the yeast at 1:3000, and the latter inhibited the bacterium at a minimal concentration of 1:20,000.

DISCUSSION OF RESULTS

Antimicrobial Studies.—Antimicrobial studies showed that, of the preservatives tested, *o*-phenylphenol was the most effective against the *Aspergillus*, *Penicillium*, and the yeast, with minimal effective concentrations of 1:21,000, 1:30,000, and 1:17,500, respectively, required for complete inhibition of growth. Butyl *p*-hydroxybenzoate at a concentration 1:22,500 was most effective against the bacterium.

A relationship of the inhibitory power of the preservatives compared on a weight to weight basis and setting the value for benzoic acid as unity is presented in Table IV.

TABLE IV.—RELATIONSHIP OF ACTIVITY OF PRESERVATIVES TESTED

Preservative	Microorganism			
	Aspergillus	*Penicillium*	Bacterium	Yeast
Benzoic acid	1	1	1	1
Sorbic acid	1	0.8	1	1
o-Phenylphenol	28	24	2.67	17.5
Esters of *p*-hydroxybenzoic acid:				
Methyl	2	1.6	0.25	1
Ethyl	3.3	3.2	0.5	2.75
Propyl	6	6.4	1.17	6.5
Butyl	8.67	8.4	4	10.5
Benzyl	12	12	7.5	17

Sorbic acid exhibited no particular advantage over benzoic acid, whereas *o*-phenylphenol had the greatest overall antimicrobial activity. It is interesting to note that the methyl, ethyl, and propyl esters were most effective against the fungi, whereas the butyl and benzyl esters were most effective against the bacterium (Table I).

General Storage Tests.—All of the chemicals tested preserved syrup, U. S. P., and diluted syrup at concentrations within the limits of their water solubility when stored in direct sun rays. When the syrups were stored in the refrigerator, sorbic acid and benzyl *p*-hydroxybenzoate were insoluble in concentrations tested. Since there was no growth in any of the refrigerated samples preserved with these chemicals, concentrations lower than those employed may be effective.

In all instances, a lower concentration of chemical was sufficient to preserve those samples stored in the refrigerator than those stored at room temperature; lower concentrations were also required for diluted syrups and proportionately lower concentrations for refrigerated diluted syrups.

In general, higher concentrations of the preservatives were required to inhibit the growth of microorganisms in syrup, U. S. P., than were determined by the antimicrobial tests on agar. In many cases, lower concentrations were effective in diluted syrup than were required in the antimicrobial studies.

Inoculated Syrups.—In all but one case, *o*-phenylphenol at a concentration 1:10,000, the growth of the *Aspergillus* upon direct inoculation of the syrups was not inhibited by the preservatives in either of the syrups under either storage condition for 60 days. These results may be attributed to the relatively high initial viable spore count.

Potentizing Action of Mixtures of *p*-Hydroxybenzoates.—A comprehensive study of the activity of mixtures of *p*-hydroxybenzoates was made to determine if mixtures of these esters exhibited a potentizing effect. Various mixtures of the five most commonly employed esters were used.

Antimicrobial Studies.—The 126 proportions of 26 mixtures of two, three, four, and five esters which were tested against four microorganisms isolated from deteriorated syrups indicated that the activity of mixtures of these esters was additive and in many cases potentizing. Nineteen of these proportions exhibited a potentiation of 10% or more when tested against an *Aspergillus*. When these same proportions were tested against the other isolates a potentiation of 10% or more was shown in four different cases against the *Penicillium* and bacterium, respectively, and in five different cases against the yeast (Table III). One proportion of a mixture of the methyl, ethyl, propyl, and butyl esters (70:10:10:10 w/w) showed a potentiation of 30% or more against all four isolates. In most instances, the other proportions showed potentiation against at least two of the isolates.

Storage Tests.—Thirty-nine of the 126 proportions tested showed a potentizing effect in syrup, U. S. P., stored in direct sun rays for 60 days. Calculations were not made for syrup, U. S. P., stored in the refrigerator or for diluted syrup (42.5% sucrose w/v) in either storage condition because minimal effective preservative concentrations were not determined due to insufficient dilution range. A potentiation of 100% was shown by a mixture of the ethyl, propyl, butyl, and benzyl esters (10:70:10:10 w/w) at concentration 1:4500. Of the proportions which showed potentiation, a mixture of the methyl, ethyl, propyl, butyl, and benzyl esters (15:15:15:15:40 w/w) had the lowest minimal effective concentration, 1:7500, and a potentiation of 20.5%.

Suggested Preservatives for Syrup, U. S. P.—If the use of a single chemical is desired as the preservative for syrup, U. S. P., *o*-phenylphenol at a concentration 1:15,000 is recommended. If the use of a *p*-hydroxybenzoate is preferred then propyl *p*-hydroxybenzoate at a concentration 1:5000 is suggested. Although the benzyl ester was effective at a concentration 1:11,000, its use is questionable for at this concentration it is insufficiently soluble below room temperature. Since a trend toward using mixtures of the *p*-hydroxybenzoates exists, it is recommended that a mixture of the five esters in the proportion 15:15:15:15:40 w/w at a concentration 1:7500 be employed.

Regardless of the chemical or mixtures of esters chosen as the preservative, the cost of preserving syrup, U. S. P., would be considerably less than one cent per gallon at the prevailing market prices for these chemicals. Further tests are suggested on the use of these chemicals in larger quantities of syrup

and for extended storage periods. Taste tests and toxicity tests (acute and chronic) should be conducted and the approval of the Food and Drug Administration obtained before some of these chemicals could be employed as preservatives for syrups.

FD&C Colors.—None of the FD&C colors tested showed activity against the molds at a concentration 1:500. Inhibition of the bacterium and yeast at minimal concentrations of 1:4700 and 1:3000, respectively, was shown by FD&C Orange No. 1; FD&C Red No. 3 inhibited the bacterium at a minimal concentration of 1:20,000.

SUMMARY

1. The activity of eight chemical preservatives was tested against four microorganisms isolated from deteriorated pharmaceutical syrups employing pure culture technique.

2. All chemicals tested effectively preserved syrup, U. S. P., when stored in the direct sun rays for 60 days.

3. The activity of mixtures of esters of *p*-hydroxybenzoic acid was shown to be additive and in some cases potentizing in both the antimicrobial studies and storage tests.

4. The use of *o*-phenylphenol and propyl *p*-hydroxybenzoate at concentrations 1:15,000 and 1:5000, respectively, and a mixture of five *p*-hydroxybenzoates at a concentration 1:7500 as preservatives for syrup, U. S. P., were recommended with reservations.

5. The FD&C colors were tested, and it was shown that Orange No. 1 was antagonistic to the bacterium and yeast, and Red No. 3 was antagonistic to the bacterium.

REFERENCES

(1) Fishburn, A. G., *Quart. J. Pharm. Pharmacol.*, **21**, 451(1948).
(2) Owen, W. L., "The Microbiology of Sugars, Syrups and Molasses," Barr-Owen Research Enterprises, New Orleans, 1949.
(3) Lord, C. F., and Husa, W. J., THIS JOURNAL, **43**, 438 (1954).
(4) Littlejohn, O. M., and Husa, W. J., *ibid.*, **44**, 305(1955).
(5) Aalto, T. R., Firman, M. C., and Rigler, N. E., *ibid.*, **42**, 449(1953).

An Investigational Study of Hydroxy Aluminum Magnesium Aminoacetate, A New Antacid*

By ARTHUR G. ZUPKO†

The physical and chemical properties of a new antacid, hydroxy aluminum magnesium aminoacetate (HAMA), are described. *In vitro* studies indicate HAMA responds rapidly to 0.1 *N* HCl and has a long-sustained buffering capacity. It neither stimulates production of gastric acidity nor permits acid rebound. It appears to be compatible with anticholinergics and possesses some antiulcer effects against gastrointestinal ulcers produced in guinea pigs by antihistamine-depot histamine injections. The compound is nonsystemic in its effects and comparatively devoid of toxicity. It meets most of the requirements for an ideal antacid and should be efficacious in peptic ulcer therapy and hyperacidity.

ATTEMPTS TO COMBINE aluminum and magnesium salts into a single chemical compound for use as an antacid have met with little success owing to the constipating effects of the former and the laxative properties of the latter. Recently a new antacid, hydroxy aluminum magnesium aminoacetate (HAMA),[1] was developed to offset these undesirable effects by utilizing balanced proportions of aluminum and magnesium. Clinical studies are now in progress to ascertain its value in peptic ulcer therapy and hyperacidity.

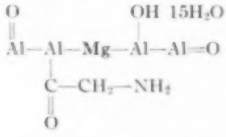
An investigational study was undertaken to determine the efficacy of this newly developed compound in meeting the requirements for an ideal antacid as set forth in a recent publication by Dale and Booth (1). Accordingly, *in vitro* studies were done to determine the comparative neutralizing capacity of the compound. In addition, its compatibility with anticholinergic agents, its antiulcer effect against antihistamine-

* Received August 8, 1955, from St. Louis College of Pharmacy and Allied Sciences, St. Louis 10, Mo.
The author wishes to thank Dr. Geo. H. Wright of Victor M. Hermelin and Company for generous supplies of Glycamine-A.M. Thanks are also due Dr. Wm. Crosson (G. D. Searle Co.) for Banthine® and Pro-Banthine®, Dr. Geo. Babcock (Schering Corp.) for Prantal®, Dr. Joe Webb (Upjohn Co.) for Pamine®, Dr. John Saunders (Ciba) for Antrenyl® and Dr. Donald Searle (Burroughs Wellcome and Co.) for Tricoloid® supplies used in this study.
† Present address: Long Island University, Brooklyn College of Pharmacy, Brooklyn 16, New York.
[1] Glycamine-A.M. brand of hydroxy aluminum magnesium aminoacetate manufactured by Victor M. Hermelin and Company, St. Louis, Mo.

depot histamine gastrointestinal ulcers produced in guinea pigs, and the toxicity of this new compound were studied.

EXPERIMENTAL

Physical and Chemical Properties.—Hydroxy aluminum magnesium aminoacetate has the following proposed structure:



Its molecular weight is 509.24 with 21.19% aluminum, 4.7% magnesium, and 2.75% nitrogen as active elements. Assays for aluminum, magnesium, and nitrogen correspond precisely with the calculated values of the proposed structural formula.

The compound is a white powder having a pleasant, slightly sweet taste. It is relatively insoluble in water and moderately soluble in dilute acids and alkalies. The compound is amphoteric in nature. Acid solutions rapidly form into a heavy, tenacious demulcent gel which is very stable and long lasting.

In Vitro Studies.—The acid consuming properties of HAMA and four other antacids were studied using a modified U. S. P. XIV method (2). One gram of active medication was used to test each of the antacids. Figure 1 shows that HAMA has an acid consuming capacity of 193 cc. The four commonly used ethical antacid preparations tested by this method have acid consuming capacities varying from 70.2–216.0 cc. It is to be noted that only Antacid A has a higher acid consuming capacity than HAMA.

These same antacids were tested for their buffering capacity by the method of Hammarlund and Rising (3). The results are shown in Fig. 2. From these data it may be observed that HAMA has a prompt response to 0.1 *N* HCl. Its buffering capacity and long-sustained action are demonstrated by the fact that the *p*H of the solution does not rise above 4.25 and is maintained within a *p*H range of 3.0–4.25 for 4½ hours. Antacid A has a relatively high buffering capacity but operates almost entirely in the alkaline range (*p*H 7.0–9.0).

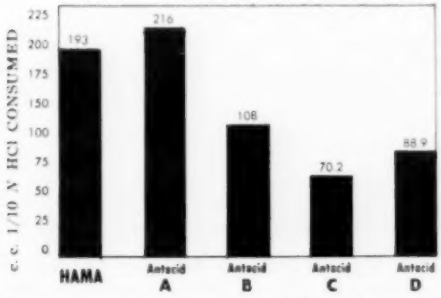


Fig. 1.—Acid consuming capacity of antacids (U.S.P. XIV method).

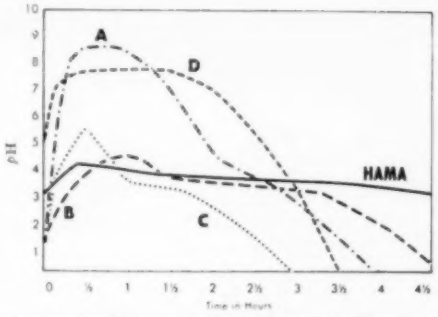


Fig. 2.—Buffering capacity of antacid (Hammarlund-Rising method).

The extremely sharp rise and fall in the curve indicates acid rebound as well as acid stimulating properties. Antacid B has a comparatively low buffering capacity but a relatively good *p*H curve for three hours. Antacid C has a very low buffering capacity, the *p*H rises and falls rapidly and its duration of action is only 1½ hours. Antacid D also has a very low buffering capacity and operates almost entirely within the alkaline range.

Adsorption Effect on Anticholinergic Drugs.—The adsorption effect of various aluminum antacids has been demonstrated by Grote and Woods (4). With the advent of newer anticholinergic agents it was thought desirable to investigate the adsorption effect of HAMA as well as other aluminum antacids upon six newer anticholinergic agents introduced since 1950.

Using a modification of the method of Grote and Woods (4) the approximate rat oral LD_{50} dose for the anticholinergic drug under test was determined, then this same dose of drug was given in admixture with the aluminum antacid to be tested in order to determine if the same lethal effect was obtained after admixture as before.

Male rats of the Sprague-Dawley strain weighing 200–300 Gm. were used throughout the study. Groups of 20 rats each were used for each of the antacids tested as well as for the determination of the approximate LD_{50} dose of the anticholinergics. The antacid-anticholinergic mixtures were prepared immediately prior to oral administration. No attempt was made to determine the effect of time upon the adsorption of the anticholinergic by the antacid via allowing the admixture to stand for several days. The dosage of each of the antacids was 1 Gm./Kg. of weight. The results are shown in Table I.

From these data it may be seen that no single anticholinergic drug tested is consistently adsorbed to a greater or lesser degree than the others by all the antacids. The greatest adsorption of the anticholinergics was shown by a freshly prepared magma made from aluminum hydroxide dried gel. The mortality was reduced to as low as 35% with an average mortality for all anticholinergics tested of 48.3%. The adsorption was much less in the case of aluminum phosphate magma, the lowest mortality being 65% with an average mortality reduction of 75%. The least adsorption was shown by both HAMA and dihydroxy aluminum aminoacetate. The average mortality reduction for the

TABLE I.—ACUTE ORAL TOXICITY OF ANTICHOLINERGIC-ANTACID MIXTURES

Anticholinergic	Dose mg./Kg.	Anticholinergic Only	$Al(OH)_3$ gel, dried	Dihydroxy Al Aminoacetate	$AlPO_4$ Magma	HAMA
Banthine	1,735	19/20[a]	11/20	18/20	15/20	18/20
		95%	55%	90%	75%	90%
Pamine	1,685	18/20	9/20	17/20	16/20	17/20
		90%	45%	85%	80%	85%
Prantal	1,260	17/20	7/20	16/20	13/20	18/20
		85%	35%	80%	65%	90%
Antrenyl	1,090	17/20	8/20	16/20	16/20	16/20
		85%	40%	80%	80%	80%
Tricoloid	1,050	19/20	13/20	17/20	14/20	18/20
		95%	56%	85%	70%	90%
Pro-Banthine	1,210	18/20	10/20	16/20	16/20	19/20
		90%	50%	80%	80%	95%

[a] Mortality is expressed as the ratio of number of rats dead in 48 hours to the number of rats tested.

TABLE II.—EFFECT OF ALUMINUM ANTACIDS ON THE DEVELOPMENT OF GASTROINTESTINAL ULCERATION

Drug and Dose Used Daily	Ulcerated Animals/ Total Animals Used	Animals Ulcerated, %	Mean Degree of Ulceration	Index of Ulceration
2 mg./Kg. Benadryl s.c.; 25 mg./Kg. depot histamine, and 5 mg./Kg. Benadryl i.m.	15/18	83.3	2.85	2.37
Aluminum hydroxide gel, dried 2 × 0.5 Gm./Kg.	9/12	75.0	2.48	1.86
Dihydroxy aluminum aminoacetate 2 × 0.5 Gm./Kg.	7/12	58.3	1.45	0.84
Aluminum Phosphate Gel, dried 2 × 0.5 Gm./Kg.	10/12	83.3	2.66	2.21
HAMA, 2 × 0.5 Gm./Kg.	7/12	58.3	1.10	0.64

former was 88.3% and the latter 83.3%. The lethal effect of Prantal® and Pro-Banthine® was actually enhanced when HAMA was administered concomitantly.

Effect on the Development of Gastrointestinal Ulcers.—Hay, *et al.* (5) produced gastrointestinal ulceration in many animal species with daily injections of histamine in oil and beeswax. Recently Harrisson, *et al.* (6) modified this procedure by producing ulcers in guinea pigs with daily subcutaneous injections of an antihistamine followed by an intramuscular injection of histamine diphosphate and an antihistamine in oil and beeswax. Several authors (7–9) have demonstrated that antihistamines offer protection against the respiratory and cardiovascular effects of histamine but are ineffective against gastrointestinal ulcer-inducing effects. Ulcer protection in guinea pigs via antihistamine-depot histamine injections as described by Harrisson, *et al.* (6) was used to test the antiulcer effect of HAMA and other aluminum antacids.

The method consisted essentially of subcutaneous daily injections to guinea pigs of 2 mg./Kg. Benadryl® followed by an intramuscular injection of 25 mg./Kg. of histamine diphospate and 5 mg./Kg. of Benadryl in beeswax and oil. The incorporation of the drugs into beeswax and oil is adequately described by Harrisson, *et al.* (6). A dose of 0.5 Gm./Kg. of a freshly prepared aqueous suspension of the antacid under test was administered orally twice daily; in the early morning and late afternoon. Those animals dying during the experiment were autopsied as soon after death as feasible. All survivors were autopsied on the tenth day and the stomach and duodenum examined for ulceration. After the establishment of the degree of ulceration (0 to 4 plus) the index of ulceration was calculated according to the method described by Pauls, *et al.* (10), viz: Index of Ulceration = mean degree of ulceration × % of group ulcerated/100. Eighteen control guinea pigs were used to ascertain the efficacy of the ulcer production method used. A total of sixty-six guinea pigs, all males, were used throughout the experiments.

Table II shows the results obtained with the controls as well as the aluminum antacids tested. From these data it may be observed that neither aluminum hydroxide nor aluminum phosphate has any influence on gastrointestinal ulceration under the experimental conditions described. In each case the mean degree of ulceration closely approximated that produced by the antihistamine-depot histamine injections. The index of ulceration for these two antacids was also quite high. Both HAMA and dihydroxy aluminum aminoacetate were somewhat more effective, each causing 25% less ulceration than the controls. Both the mean degree of ulceration and the index of ulceration were higher for dihydroxy aluminum aminoacetate than HAMA. The index of ulceration is highest with aluminum phosphate and lowest with HAMA.

It is highly likely that different dosage levels would produce more variable results, but it was felt that the utilization of higher levels would not be feasible.

LD_{50} Determination.—Male rats of the Sprague-Dawley strain weighing 250–350 Gm. were administered HAMA orally in doses up to 3,500 mg./Kg. with few deaths encountered. Owing to the large

volume of antacid required, it was deemed impractical to attempt administration of dosages in excess of 3,500 mg./Kg.

Chronic Toxicity.—Thirty male rats of the Sprague-Dawley strain were divided equally into two groups. Each group received daily oral doses of HAMA, 0.5 Gm./Kg. and 1.0 Gm./Kg. respectively. None of the test animals exhibited any evidence of systemic toxicity. The rats were sacrificed at the end of forty days and sections of the liver, kidney, stomach, intestine, and spleen were cut and stained according to the method of Zupko and Edwards (11). Histological examination of these sections revealed no discernible pathological changes.

During the course of the chronic toxicity experiment the rats were examined periodically to determine any changes in the acid-base balance. No significant changes were observed during the course of the experiment.

DISCUSSION

Although *in vitro* studies indicate that Antacid A has a higher acid consuming capacity than HAMA, it should be pointed out that Antacid A operates in an alkaline *p*H range. It is generally agreed that this range may cause impaired digestion as well as acid rebound and alkalosis. HAMA operates entirely within the ideal range of *p*H 3.0–5.0, insuring optimal conditions for proper digestion.

Further evidence of the high acid consuming capacity of HAMA is indicated by noting that $NaHCO_3$ consumes 120 cc. of 0.1 *N* HCl, Mg trisilicate 155 cc., and $Al(OH)_3$ 235 cc., as compared to HAMA which consumes 193 cc. of 0.1 *N* HCl when tested by the U. S. P. XIV method (2).

The use of anticholinergic agents with aluminum antacids for control of ulcer pain is a common procedure in therapeutics. Investigation of the adsorption of these drugs is, therefore, not without merit. Under the experimental conditions stated it would appear that both HAMA and dihydroxy aluminum aminoacetate show little adsorptive effects. However, such is not the case with $Al(OH)_3$ and $AlPO_4$, which show considerable adsorptive power. It is highly likely that this same effect is exhibited in the human being when an anticholinergic agent is administered concomitantly with large doses of one or the other of these antacids. Conceivably such an effect would require closer supervision of the amounts of these antacids consumed or a more stringent regulation of anticholinergic dosage.

The influence exerted by the aluminum antacids alone on gastrointestinal ulceration produced by the antihistamine-depot histamine method is not striking. One cannot overlook the fact, however, that both HAMA and dihydroxy aluminum aminoacetate reduce the incidence of ulceration by 25%, indicating partial effectiveness as antiulcer agents. Certainly they appear to be superior to both the hydroxide and phosphate forms of aluminum.

For all practical purposes HAMA appears to be devoid of toxicity in large doses and when used over a long period of time.

SUMMARY

1. The physical and chemical properties of a new antacid, hydroxy aluminum magnesium aminoacetate (HAMA), have been described.

2. *In vitro* studies indicate that the compound has a high acid consuming capacity. It has a prompt response to 0.1 *N* HCl and a long-sustained buffering capacity. Under the experimental conditions described it maintains a *p*H range of 3.0 to 4.25 for four and one-half hours. It neither stimulates production of gastric acidity nor permits acid rebound.

3. HAMA appears to be compatible with the newer anticholinergic agents as indicated by its low adsorptive power.

4. HAMA affords partial protection against gastrointestinal ulcers produced by antihistamine-depot histamine injections in guinea pigs, as evidenced by a 25 per cent reduction in the incidence of ulcer formation in these animals.

5. Toxicity studies indicate the compound is comparatively devoid of toxicity and is completely nonsystemic in its effects.

6. HAMA meets most of the requirements for an ideal antacid. It would appear to be well adapted for the treatment of peptic ulcers and hyperacidity.

REFERENCES

(1) Dale, J. K., and Booth, R. E., THIS JOURNAL, **44,** 170(1955).
(2) United States Pharmacopeia, 14th rev., Mack Publishing Company, Easton, Pa., 1950, p. 29.
(3) Hammarlund, E. R., and Rising, L. W., THIS JOURNAL, **38,** 586(1949).
(4) Grote, I. W., and Woods, M., *ibid.*, **42,** 319(1953).
(5) Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gynecol. Obstet.*, **75,** 170(1942).
(6) Harrisson, J. W. E., Packman, E. W., Guth, P. S., Back, N., and Chernick, W. S., THIS JOURNAL, **44,** 123(1955).
(7) Friesen, S. R., Baronofsky, I. D., and Wangensteen, O. H., *Proc. Soc. Exptl. Biol. Med.*, **63,** 23(1946).
(8) Crane, J. T., Lindsay, S., and Dailey, N. E., *Am. J. Digest. Diseases*, **14,** 56(1947).
(9) Ambrus, J. L., Ambrus, C. M., and Harrisson, J. W. E., *Amer. J. Physiol.*, **173,** 95(1953).
(10) Pauls, F., Wick, A. M., and McKay, E. M., *Gastroenterology*, **8,** 774(1947).
(11) Zupko, A. G., and Edwards, L. D., THIS JOURNAL, **37,** 124(1949).

TABLE I.—ACUTE ORAL TOXICITY OF ANTICHOLINERGIC-ANTACID MIXTURES

Anticholinergic	Dose mg./Kg.	Anticholinergic Only	$Al(OH)_3$ gel, dried	Dihydroxy Al Aminoacetate	$AlPO_4$ Magma	HAMA
Banthine	1,735	19/20[a]	11/20	18/20	15/20	18/20
		95%	55%	90%	75%	90%
Pamine	1,685	18/20	9/20	17/20	16/20	17/20
		90%	45%	85%	80%	85%
Prantal	1,260	17/20	7/20	16/20	13/20	18/20
		85%	35%	80%	65%	90%
Antrenyl	1,080	17/20	8/20	16/20	16/20	16/20
		85%	40%	80%	80%	80%
Tricoloid	1,050	19/20	13/20	17/20	14/20	18/20
		95%	56%	85%	70%	90%
Pro-Banthine	1,210	18/20	10/20	16/20	16/20	19/20
		90%	50%	80%	80%	95%

[a] Mortality is expressed as the ratio of number of rats dead in 48 hours to the number of rats tested.

TABLE II.—EFFECT OF ALUMINUM ANTACIDS ON THE DEVELOPMENT OF GASTROINTESTINAL ULCERATION

Drug and Dose Used Daily	Ulcerated Animals/ Total Animals Used	Animals Ulcerated, %	Mean Degree of Ulceration	Index of Ulceration
2 mg./Kg. Benadryl s.c.; 25 mg./Kg. depot histamine, and 5 mg./Kg. Benadryl i.m.	15/18	83.3	2.85	2.37
Aluminum hydroxide gel, dried 2 × 0.5 Gm./Kg.	9/12	75.0	2.48	1.86
Dihydroxy aluminum aminoacetate 2 × 0.5 Gm./Kg.	7/12	58.3	1.45	0.84
Aluminum Phosphate Gel, dried 2 × 0.5 Gm./Kg.	10/12	83.3	2.66	2.21
HAMA, 2 × 0.5 Gm./Kg.	7/12	58.3	1.10	0.64

former was 88.3% and the latter 83.3%. The lethal effect of Prantal® and Pro-Banthine® was actually enhanced when HAMA was administered concomitantly.

Effect on the Development of Gastrointestinal Ulcers.—Hay, *et al.* (5) produced gastrointestinal ulceration in many animal species with daily injections of histamine in oil and beeswax. Recently Harrisson, *et al.* (6) modified this procedure by producing ulcers in guinea pigs with daily subcutaneous injections of an antihistamine followed by an intramuscular injection of histamine diphosphate and an antihistamine in oil and beeswax. Several authors (7–9) have demonstrated that antihistamines offer protection against the respiratory and cardiovascular effects of histamine but are ineffective against gastrointestinal ulcer-inducing effects. Ulcer protection in guinea pigs via antihistamine-depot histamine injections as described by Harrisson, *et al.* (6) was used to test the antiulcer effect of HAMA and other aluminum antacids.

The method consisted essentially of subcutaneous daily injections to guinea pigs of 2 mg./Kg. Benadryl® followed by an intramuscular injection of 25 mg./Kg. of histamine diphospate and 5 mg./Kg. of Benadryl in beeswax and oil. The incorporation of the drugs into beeswax and oil is adequately described by Harrisson, *et al.* (6). A dose of 0.5 Gm./Kg. of a freshly prepared aqueous suspension of the antacid under test was administered orally twice daily; in the early morning and late afternoon. Those animals dying during the experiment were autopsied as soon after death as feasible. All survivors were autopsied on the tenth day and the stomach and duodenum examined for ulceration. After the establishment of the degree of ulceration (0 to 4 plus) the index of ulceration was calculated according to the method described by Pauls, *et al.* (10), viz: Index of Ulceration = mean degree of ulceration × % of group ulcerated/100. Eighteen control guinea pigs were used to ascertain the efficacy of the ulcer production method used. A total of sixty-six guinea pigs, all males, were used throughout the experiments.

Table II shows the results obtained with the controls as well as the aluminum antacids tested. From these data it may be observed that neither aluminum hydroxide nor aluminum phosphate has any influence on gastrointestinal ulceration under the experimental conditions described. In each case the mean degree of ulceration closely approximated that produced by the antihistamine-depot histamine injections. The index of ulceration for these two antacids was also quite high. Both HAMA and dihydroxy aluminum aminoacetate were somewhat more effective, each causing 25% less ulceration than the controls. Both the mean degree of ulceration and the index of ulceration were higher for dihydroxy aluminum aminoacetate than HAMA. The index of ulceration is highest with aluminum phosphate and lowest with HAMA.

It is highly likely that different dosage levels would produce more variable results, but it was felt that the utilization of higher levels would not be feasible.

LD_{50} Determination.—Male rats of the Sprague-Dawley strain weighing 250–350 Gm. were administered HAMA orally in doses up to 3,500 mg./Kg. with few deaths encountered. Owing to the large

volume of antacid required, it was deemed impractical to attempt administration of dosages in excess of 3,500 mg./Kg.

Chronic Toxicity.—Thirty male rats of the Sprague-Dawley strain were divided equally into two groups. Each group received daily oral doses of HAMA, 0.5 Gm./Kg. and 1.0 Gm./Kg. respectively. None of the test animals exhibited any evidence of systemic toxicity. The rats were sacrificed at the end of forty days and sections of the liver, kidney, stomach, intestine, and spleen were cut and stained according to the method of Zupko and Edwards (11). Histological examination of these sections revealed no discernible pathological changes.

During the course of the chronic toxicity experiment the rats were examined periodically to determine any changes in the acid-base balance. No significant changes were observed during the course of the experiment.

DISCUSSION

Although *in vitro* studies indicate that Antacid A has a higher acid consuming capacity than HAMA, it should be pointed out that Antacid A operates in an alkaline *p*H range. It is generally agreed that this range may cause impaired digestion as well as acid rebound and alkalosis. HAMA operates entirely within the ideal range of *p*H 3.0–5.0, insuring optimal conditions for proper digestion.

Further evidence of the high acid consuming capacity of HAMA is indicated by noting that $NaHCO_3$ consumes 120 cc. of 0.1 *N* HCl, Mg trisilicate 155 cc., and $Al(OH)_3$ 235 cc., as compared to HAMA which consumes 193 cc. of 0.1 *N* HCl when tested by the U. S. P. XIV method (2).

The use of anticholinergic agents with aluminum antacids for control of ulcer pain is a common procedure in therapeutics. Investigation of the adsorption of these drugs is, therefore, not without merit. Under the experimental conditions stated it would appear that both HAMA and dihydroxy aluminum aminoacetate show little adsorptive effects. However, such is not the case with $Al(OH)_3$ and $AlPO_4$, which show considerable adsorptive power. It is highly likely that this same effect is exhibited in the human being when an anticholinergic agent is administered concomitantly with large doses of one or the other of these antacids. Conceivably such an effect would require closer supervision of the amounts of these antacids consumed or a more stringent regulation of anticholinergic dosage.

The influence exerted by the aluminum antacids alone on gastrointestinal ulceration produced by the antihistamine-depot histamine method is not striking. One cannot overlook the fact, however, that both HAMA and dihydroxy aluminum aminoacetate reduce the incidence of ulceration by 25%, indicating partial effectiveness as antiulcer agents. Certainly they appear to be superior to both the hydroxide and phosphate forms of aluminum.

For all practical purposes HAMA appears to be devoid of toxicity in large doses and when used over a long period of time.

SUMMARY

1. The physical and chemical properties of a new antacid, hydroxy aluminum magnesium aminoacetate (HAMA), have been described.

2. *In vitro* studies indicate that the compound has a high acid consuming capacity. It has a prompt response to 0.1 *N* HCl and a long-sustained buffering capacity. Under the experimental conditions described it maintains a *p*H range of 3.0 to 4.25 for four and one-half hours. It neither stimulates production of gastric acidity nor permits acid rebound.

3. HAMA appears to be compatible with the newer anticholinergic agents as indicated by its low adsorptive power.

4. HAMA affords partial protection against gastrointestinal ulcers produced by antihistamine-depot histamine injections in guinea pigs, as evidenced by a 25 per cent reduction in the incidence of ulcer formation in these animals.

5. Toxicity studies indicate the compound is comparatively devoid of toxicity and is completely nonsystemic in its effects.

6. HAMA meets most of the requirements for an ideal antacid. It would appear to be well adapted for the treatment of peptic ulcers and hyperacidity.

REFERENCES

(1) Dale, J. K., and Booth, R. E., THIS JOURNAL, **44**, 170(1955).
(2) United States Pharmacopeia, 14th rev., Mack Publishing Company, Easton, Pa., 1950, p. 29.
(3) Hammarlund, E. R., and Rising, L. W., THIS JOURNAL, **38**, 586(1949).
(4) Grote, I. W., and Woods, M., *ibid.*, **42**, 319(1953).
(5) Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gynecol. Obstet.*, **75**, 170(1942).
(6) Harrisson, J. W. E., Packman, E. W., Guth, P. S., Back, N., and Chernick, W. S., THIS JOURNAL, **44**, 123(1955).
(7) Friesen, S. R., Baronofsky, I. D., and Wangensteen, O. H., *Proc. Soc. Exptl. Biol. Med.*, **63**, 23(1946).
(8) Crane, J. T., Lindsay, S., and Dailey, N. E., *Am. J. Digest. Diseases*, **14**, 56(1947).
(9) Ambrus, J. L., Ambrus, C. M., and Harrisson, J. W. E., *Amer. J. Physiol.*, **173**, 95(1953).
(10) Pauls, F., Wick, A. M., and McKay, E. M., *Gastroenterology*, **8**, 774(1947).
(11) Zupko, A. G., and Edwards, L. D., THIS JOURNAL, **37**, 124(1949).

Modern Ointment Base Technology II.*

Comparative Evaluation of Bases

By MARGARET N. MUTIMER, CHARLES RIFFKIN, JOHN A. HILL, MURRAY E. GLICKMAN, and GILMAN N. CYR

Selection of the base for an ointment should be made only after comparative evaluations of many factors, among which are: (*a*) drug-release characteristics, (*b*) consistency and body as defined by viscosity, tube extrusion pressure, penetrometer readings, spreadability, and effect of increased solids, (*c*) physical and chemical stability, and (*d*) handling and manufacturing characteristics. Methods for measuring some of these properties are described, and by such methods commonly used ointment bases are compared.

AN OINTMENT BASE should always complement the medication which it carries, always adding to the therapeutic effectiveness of the medicament, never detracting from it. However, what may be an excellent base for a given drug may actually be detrimental to the effectiveness of another. In order to arrive at a satisfactory combination of drug and base, it is necessary to be able to specify those properties of the base which would be most desirable for a given situation.

The properties of ointment bases can be divided into four general categories. First, those which exert an effect on the therapeutic manifestations of the active ingredient; second, those which have an effect on patient acceptance of the finished product; third, those which control the stability aspects; and fourth, those affecting the compounding and manufacturing of finished products.

From the standpoint of therapeutics, one of the most important attributes of an ointment base is its ability to release the active ingredient which it carries. This is true except when the ointment is applied for physical protection or for some purely mechanical purpose. If the ointment has antibacterial or pharmacologic activity, such activity should be made available to the substrate being treated. However, there are certain instances where it may be desirable to prolong the release of the drug from the base. Also an ointment base should never irritate either the intact skin or the abraded skin and neither should it retard the normal healing process.

The patient may judge an ointment on such points as ease of extrusion from a tube, ease of spreading, odor, staining properties, removal from the site of application, and other similar factors falling within the realm of elegance.

The pharmacist is concerned mainly with the range of compatibility of any one ointment base as well as with the stability of the base and of ointments made from it. In addition, the pharmacist prefers a base which is economical and easy to handle and which is a good levigating agent.

In our work with ointment bases, we have attempted to measure a number of these properties in order that we might evaluate properly the many ointment bases available. Such an evaluation serves as a basis for the logical selection of an ointment base.

MEASUREMENT OF DRUG RELEASE

Perhaps the most important function of an ointment base is the control that it exerts over the therapeutic activity of the drug which it carries. It is generally accepted that for a drug to be effective it must first be released from its carrier in some manner. *In vitro* drug release tests although not necessarily reflecting *in vivo* activity, can measure the rate and extent of this release. A number of methods have been reported for determining the release of drugs from ointments. These are known to those familiar with the literature on ointments.

Dialysis Method.—Jurist (1) has described a method for measuring the ion-exchange capacity of a resin incorporated in an ointment base. The method is based on an exchange, through a semipermeable membrane, of hydrogen ions on the resin for sodium ions in a solution of sodium citrate. This method has been modified and used satisfactorily for comparing the relative effectiveness of Amberlite® XE-64[1] (325 mesh) when incorporated at the rate of 15% in either white petrolatum, Plastibase®,[2] or a washable, emulsion-type base. The apparatus used is shown and described in Fig. 1.

About 10 cc. of ointment was spread evenly in the depression on the bottom plate and leveled exactly with a straight-edged scraper. A sheet

* Received May 5, 1955, from the Squibb Institute for Medical Research, New Brunswick, N. J.

The authors wish to acknowledge the valuable assistance given by Dr. N. Coy and her group in conducting the spectrophotometric analyses and the generous cooperation of Dr. C. Bickford in the development of some of the apparatus.

[1] Carboxylic ion-exchange resin, Rohm and Haas, Philadelphia, Pa.

[2] Squibb hydrocarbon gel; Plastibase is a Squibb Trademark.

of semipermeable membrane[3] of 175-mm. diameter was placed over the bottom plate and the ointment. Good contact between the ointment and membrane was insured by pressing down with the top plate. The center plate and the top plate were put into position and the unit was then clamped together as shown in Fig. 1. When thus assembled, 50 cc. of a

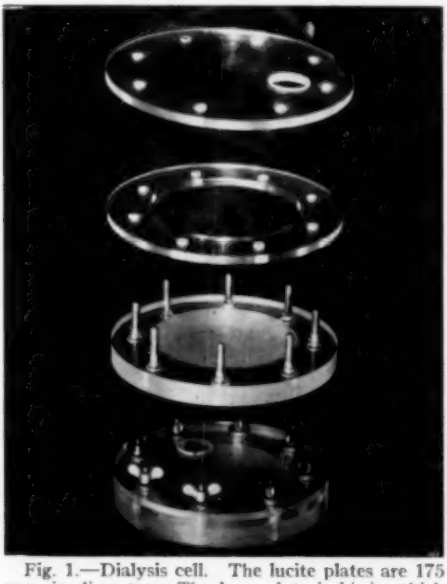


Fig. 1.—Dialysis cell. The lucite plates are 175 mm. in diameter. The base plate is $^5/_8$ in. thick and the well in the center of its upper surface is 1 mm. in depth and 112.8 mm. in diameter. The well has a capacity of 10 cc. and a surface area of about 100 cm^2. The center and top plates are $^1/_4$ in. thick. The internal diameter of the center plate is also 112.8 mm. The hole in the top plate is $1^1/_8$ in. in diameter and its outer edge is flush with the inner edge of the center plate. A sheet of dialyzing membrane (not shown), when placed between the base plate and center plate, separates the ointment in the base plate from the solution in the reservoir formed by the center plate. The top plate serves as a cover through the hole in which samples of solution may be removed. The assembled cell is shown at the bottom.

freshly prepared 5% (w/v) aqueous solution of sodium citrate (adjusted to *p*H 11.5 ± 0.05 with sodium hydroxide) was introduced into the cell through the hole in the top plate. As sodium ions in the citrate solution were exchanged for hydrogen ions on the resin, the *p*H of the citrate solution was reduced. At 15-minute intervals the cell was rotated gently on a horizontal plane to homogenize the citrate solution; a sample of 1 cc. of solution was withdrawn through the hole in the top plate and transferred to a micro cup and its *p*H determined on a Beckman model G *p*H meter. The sample was immediately returned to the dialyzer and the cell rotated to mix well. As a means of visualizing the change in *p*H, 2 cc. of Grammercy universal indicator solution may be added to the citrate solution initially and color changes observed. The results obtained with each of the three ointments are given in Fig. 2.

As may be expected from its aqueous nature, the emulsion-type base promoted the most rapid reduction in *p*H. However, clinically, this product was shown to be irritating (2) probably because of this very rapid activity. The petrolatum ointment apparently holds the resin occlusively as shown by the insignificant drop in *p*H of the citrate solution. The ointment in Plastibase shows a gradual, but nevertheless effective, release of exchange capacity and also this product was much less irritating than the hydrophilic ointment.

Extraction Method No. 1.—The ability of an ointment to release the active ingredient which it carries can also be measured by a method based on extraction. The apparatus shown and described in Fig. 3 has been used for determining the relative amount of a local anesthetic (Intracaine,[4] free base) released from either white petrolatum or Plastibase with and without a neomycin-gramicidin component.

Each tray was filled with about 5 Gm. of ointment and leveled with a straight-edged scraper. The filled trays were suspended in a 400-cc. beaker containing 250 cc. of phosphate buffer.[5] The solution in the beaker was agitated with a magnetic stirrer. The extraction was conducted at room temperature. Portions of 10 cc. of the buffer solution were removed at intervals, filtered, and the absorbance read on a Beckman spectrophotometer at a wavelength of

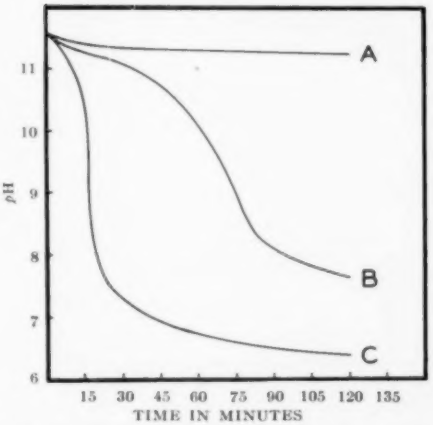


Fig. 2.—Decrease in *p*H of alkaline sodium citrate solution exposed for varying periods of time to 15 per cent ointments of Amberlite® XE-64 in white petrolatum (A), Plastibase (B), and a washable, emulsion-type base (C).

[3] Cellophane No. 300 pt. E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

[4] Intracaine is a Squibb Trademark.

[5] Disodium hydrogen phosphate, anhydrous, 0.9925 Gm.; potassium dihydrogen phosphate, 0.90 Gm.; distilled water, to make 100.0 cc. (*p*H = 6.8).

260 mμ. The values obtained were corrected for volume changes incurred by removing samples. The relative rate of extraction was calculated as the ratio of the observed absorbances. The results of the experiment are given in Table I.

TABLE I.—ABSORBANCE READINGS OBSERVED ON PHOSPHATE BUFFER SOLUTIONS (*p*H 6.8) CONTAINING INTRACAINE EXTRACTED FROM WHITE PETROLATUM (D), PLASTIBASE (E), WHITE PETROLATUM CONTAINING NEOMYCIN AND GRAMICIDIN (F), AND PLASTIBASE CONTAINING NEOMYCIN AND GRAMICIDIN (G)

Elapsed Time in Minutes	Corrected[a] Absorbance Readings		Ratio of Corrected[a] Absorbance Readings (Plastibase/White Petrolatum)
	D	E	
15	0.204	0.615	3.0
30	0.238	0.826	3.5
60	0.340	1.13	3.3
120	0.458	1.65	3.6
	F	G	
15	0.170	0.575	3.4
30	0.223	0.758	3.4
60	0.268	1.076	4.0
120	0.363	1.50	4.1

[a] Corrected to 250 cc. of solution.

The data show clearly that there is a decidedly greater total amount of Intracaine base extracted from Plastibase than from petrolatum and that the rate of release from Plastibase is more rapid than from petrolatum. Apparently the presence of neomycin (2.5 mg./Gm.) and gramicidin (0.25 mg./Gm.) has little effect on the behavior of the ointments in this test.

Extraction Method No. 2.—Another extraction method has been devised for determining the release of a water-soluble substance from a water-insoluble ointment base. This particular method has been used to determine the release of salicylic acid from Whitfield's ointment prepared with various bases. The method is a modification of one described by Foster, *et al.* (3).

A quantity of approximately 200 mg. of ointment was placed in the internal depression of a standard 20-mm. rubber stopper for multiple dose vials. The depression was completely filled and the surface leveled by drawing a straight edge over the surface. Ten cubic centimeters of 1:1000 hydrochloric acid was introduced into a multiple-dose vial of about 15-cc. capacity. The cap, with the ointment in its depression was then placed in position in the mouth of the bottle. The unit was placed horizontally in a 280 oscillations-per-minute clinical pipet shaker (Fisher Model 11 M, Serial No. 97) and shaken for a predetermined period of time. The extraction was conducted at room temperature. The solution was filtered and its salicylic acid content determined by measuring the density at 295 mμ on a Carey recording spectrophotometer. Blanks were prepared by shaking the base in the same manner and at the same time. The blank readings were subtracted from the sample readings to yield the final results.

Six per cent salicylic acid and 12% benzoic acid were incorporated in white petrolatum, U. S. P., Plastibase, Plastibase hydrophilic, and petrolatum-sorbitan sesquioleate (absorption type base), the latter two bases containing 40% water, previously added. These ointments were treated as described above and the amount of salicylic acid extracted was calculated as the per cent of total salicylic acid available from the amount (200 mg.) of ointment tested. The results are presented in Fig. 4. The data agree closely with what is generally accepted as the behavior of such bases. White petrolatum shows the lowest rate of release and the lowest total amount of salicylic acid released during the four-hour extraction period. Water was added to the absorption type bases because these bases are designed to allow for the incorporation of water and are normally used as such. These w/o emulsions provide for a greater release of salicylic acid than do hydrophobic bases. It is interesting to note that in the case of the Plastibase and Plastibase hydrophilic not only is there a greater release of salicylic acid than from white petrolatum and petrolatum-sorbitan sesquioleate, respectively, but the release of salicylic acid has not leveled off even after four hours. This can probably be attributed to the unique structure of the hydrocarbon gel which allows constant migration of oil within the matrix presenting a continually changing interface (4).

This method of test can be adapted to provide for a very rapid, semiquantitative comparison of a number of solutions. If the extractable ingredient is colored or can be developed into a color when in solution, a visual comparison of the color of these solutions is a good indication of the comparative degree of extraction. This eliminates many of the details necessary for a more accurate comparison. In the case of the salicylic acid extracts, for example,

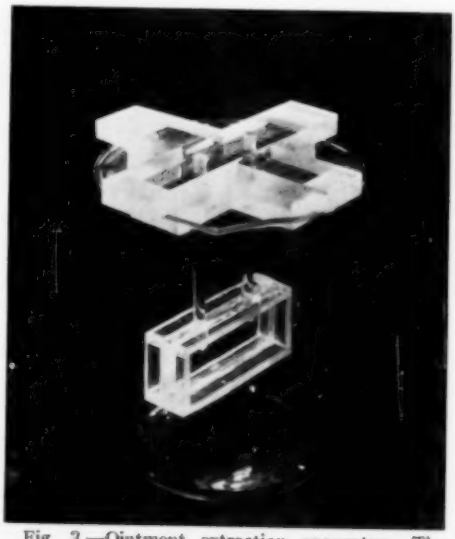


Fig. 3.—Ointment extraction apparatus. The trays which hold the ointment are back to back and can be suspended in a beaker containing an extraction liquid. Made from 1/4-in. lucite stock, each tray is 5 x 1.9 x 0.5 cm.

ferric chloride T. S. can be added to the acidified solution in the vial and its violet color compared with others similarly obtained.

Whitfield's ointments were prepared in eight different bases and extracted at room temperature for 50 minutes as described. Three drops of ferric chloride T. S. were added to each bottle to develop the violet color. The colored solutions have been photographed and are reproduced in black and white in Fig. 5. Although lacking in color, the differences in intensity are still apparent.

The value of this rapid color test can be appreciated by comparing the color intensity of the solutions extracted from white petrolatum and from Plastibase with the spectrophotometric data for these same solutions plotted in Fig. 4. This simple and rapid color test gives a very good indication of the comparative degree of extraction.

Discussion.—Each of the three methods discussed has certain advantages and certain limitations. All are fairly rapid, permitting the evaluation of many bases rather easily. All methods depend upon some degree of water solubility of the active ingredient. The dialysis method cannot be used, of course, when the active ingredient is a large molecule which is nondialyzable. In such cases, the extraction methods should be used. The extraction methods cannot be used when the bases are water soluble, because by the very nature of the apparatuses, the entire ointment, the base as well as the active ingredient, is taken up by the solution. With water-soluble bases, the dialysis method is more realistic.

That *in vitro* drug release tests have some degree of validity is shown by a comparison of some of the results obtained by these tests with the actual clinical behavior of the ointments. It has already been shown that when skin irritation is used as a criterion (2) the ion-exchange resins behaved exactly as might have been predicted from the laboratory data. In addition, 3% ointments of salicylic acid in Plastibase hydrophilic, white petrolatum, petrolatum-cholesterol, polyethylene glycol ointment, and in a washable base were applied to the back of a volunteer and left in place for 24 hours (5). Only the Plastibase hydrophilic and the petrolatum-cholesterol ointments gave a positive reaction. The tests with white petrolatum and the two water-dispersible bases were negative. A comparison of these clinical findings with the data shown in Fig. 4 and 5 indicate good agreement, except in the case of the water-dispersible bases. This serves to emphasize the better suitability of the dialysis method for measuring release from washable bases.

Fig. 5.—Intensity of color developed with ferric chloride on salicylic acid extracted from white petrolatum, U. S. P. (1), Plastibase (2), petrolatum-cholesterol (3), petrolatum-sorbitan sesquioleate (4), hydrophilic petrolatum, U. S. P. (5), Plastibase hydrophilic (6), hydrophilic ointment, U. S. P. (7), and polyethylene glycol ointment, U. S. P. (8).

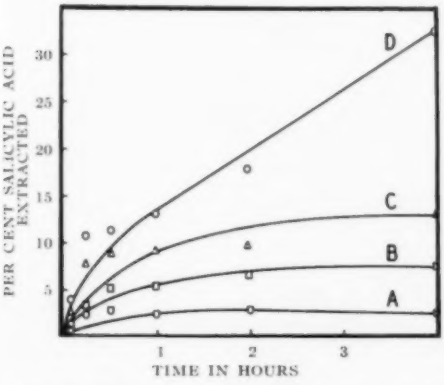


Fig. 4.—Per cent of available salicylic acid extracted from Whitfield's ointment in white petrolatum (A), Plastibase (B), petrolatum-sorbitan sesquioleate with 40% water (C), and Plastibase hydrophilic with 40% water (D).

MEASUREMENTS OF CONSISTENCY AND BODY

To a considerable extent, the utility and acceptability of an ointment base depend on consistency. Consistency may have an effect on the release of active ingredient from an ointment, the softer ointments showing usually the greater release (6). Consistency also exerts an effect on spreadability, tube extrusion pressure, and capacity for solids. Presented here are attempts to devise comparative methods of measuring these factors effectively.

Test for Spreadability.—That an ointment should spread easily is important for patient acceptance of the product. If an ointment spreads easily without the need for much pressure its application to painful areas will be much more comfortable. This is particularly true where it is necessary that the ointment be rubbed in well in order to insure maximum contact. The pharmacist considers an ointment base showing high resistance to spread a poor levi-

gating agent because much of the work input is lost in overcoming the resistance of the base. To spread well, an ointment should behave like a good lubricant exhibiting maximum "slip" and minimum "drag." On this basis, the force necessary to pull one surface over another surface both being separated by an ointment, appears to be a reasonable method for measuring spreadability.

The apparatus used in these spreadability measurements is shown in Fig. 6. An excess of the ointment is placed between two slides. A 1,000-Gm. weight is allowed to rest on the slides for five minutes to expel air between the slides and to provide for a uniform film of ointment. Excess ointment is carefully scraped off the edges of the slides. The bottom slide is anchored and the top slide subjected to a pull of 80 Gm. The time in seconds required to separate completely the slides is noted. Less time indicates more slip and better spreadability of the ointment base. The results obtained in comparing a number of ointment bases are shown in Table II.

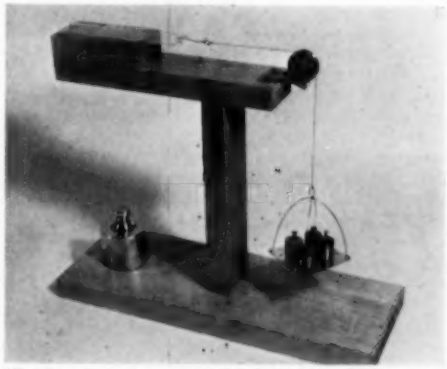


Fig. 6.—Apparatus used for measuring the spreadability of ointments.

TABLE II.—TIME IN SECONDS REQUIRED FOR A PULL OF 80 GM. TO PULL ONE GLASS SLIDE OVER THE SURFACE OF ANOTHER SEPARATED BY A FILM OF OINTMENT

Base	Time (Sec.)[a]
Plastibase	3.2
White petrolatum, U. S. P.	22
Plastibase hydrophilic	5.3
Hydrophilic petrolatum	53
Petrolatum-cholesterol	11.1
Petrolatum-sorbitan sesquioleate	12.8
Wool fat	After 3 min.—no slip

[a] Average of four determinations.

From Table II, it can be seen that Plastibase shows the least resistance to the separation of the slides. White petrolatum, on the other hand, shows a much greater resistance. This test indicates that of the absorption-type bases, the most difficult to spread is the hydrophilic petrolatum, U. S. P. Plastibase hydrophilic is the easiest to spread in the group of absorption-type bases.

Tube Extrusion Measurements.—The advantages of packaging ointments in tubes are obvious. But when packaged in a tube, an ointment should not be so soft as to run out when the cap is removed and neither should it be so stiff as to require much pressure for extrusion. This latter is particularly true for ophthalmic ointments and for veterinary ointments for the treatment of mastitis where the tube orifice is very small. It is also true for ointments which may have to be stored in a cool place. It would therefore seem reasonable to consider the ease of extrusion from a tube as a criterion for evaluating consistency or body.

Tube extrusion pressure can be expressed as the weight in grams which will extrude a ribbon of ointment 0.5 cm. long in 10.0 seconds from a tube of any standardized dimension and orifice. The apparatus used in conducting this test is shown in Fig. 7. In conducting the test, a closed tube containing the ointment is pressed firmly at the crimped end and a clamp is applied to prevent any roll-back. The cap is removed and the ointment extrudes until the pressure is dissipated. The tube is placed on the base and centered under the plunger, and the plunger is gently lowered until flush with the upper surface of the tube. The plunger is locked in place with the set screw while weights are introduced on the weighing pan.

The set screw is released and observation is made as to whether or not the weight extrudes the required 0.5 cm. ribbon of ointment in 10.0 seconds. Adjustments in weight are made until the conditions of the test are met. The results are recorded as extrusion pressure in grams. Conditions such as tube size, orifice length, internal diameter of orifice, and tube composition must be stated as these all have an effect on extrusion pressure.

Fig. 7.—Apparatus for determining tube extrusion pressure.

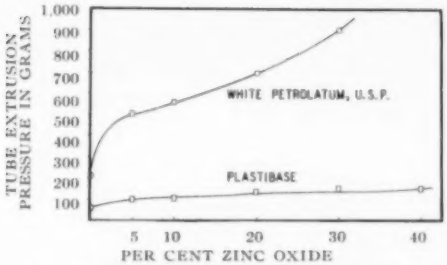


Fig. 8.—Effect on tube extrusion pressure produced by adding increments of zinc oxide to white petrolatum and to Plastibase.

The tube extrusion tests were conducted on regular tin tubes ($^7/_8$ x $4^1/_2$ in.) the nozzles of which are $^3/_{16}$ in. long with an internal orifice diameter of $^3/_{16}$ in. When tested in this manner, Lassar's paste requires a pressure of 1100 Gm. for extrusion. When Lassar's paste solids are incorporated in Plastibase, a pressure of only 200 Gm. is sufficient for extrusion.

Capacity for Solids.—Although the consistency of a base is important, of even greater importance is the consistency of ointments prepared from the base. An ointment base which maintains good consistency even with a high solids content is much more versatile than one which becomes hard when similarly loaded with solids. To demonstrate this point, increments of zinc oxide were added to two bases. The resulting ointments were packaged in standard tin tubes and extrusion pressures determined as described above. These pressures are plotted in Fig. 8. Concentrations of zinc oxide greater than 30% in white petrolatum, U. S. P. were so stiff and unmanageable as to be excluded from this test. However, although not shown in the plot, a 50% ointment of zinc oxide in Plastibase is heavy but still manageable. The data indicate that Plastibase, *per se*, is softer than white petrolatum, U. S. P. It can also be seen that Plastibase maintains a fairly uniform consistency with increasingly higher solids content. In the case of white petrolatum, U. S. P., tube extrusion pressure rises rapidly with increasing solids content.

STABILITY

The stability of ointment bases and ointments is of particular importance to the pharmaceutical manufacturer. Manufactured products are often exposed to extremes of temperature during shipment and in storage. Although the average time elapsing between manufacturing and consumption is less than a year, it is often longer (7). Naturally, the manufacturer strives to distribute products which will have a long shelf life in order that the patient will be provided with a product of consistent quality, regardless of age and storage history. Recognizing this, the pharmaceutical manufacturer should consider those properties of the base which exert an effect on the stability of the finished product.

For these reasons petrolatum and its mixtures with mineral oil and paraffin wax have always been the most dependable bases for ointments for wide distribution. Emulsified bases, glycol type bases, and bases containing animal and vegetable fats have certain desirable properties but are not so stable as petrolatum bases. However, petrolatum and its mixtures with oil and wax are easily influenced by temperature. Figure 9 demonstrates the effect of exposing 5% ointments of ammoniated mercury to a temperature of 48° overnight.

Ointments stored in very cold temperatures may become quite stiff and be difficult to extrude from the tube and to apply. An ointment which maintains its normal softness under such conditions is naturally more desirable. Figure 10 shows the appearance of 20% zinc oxide ointments kept at 5°, room temperature, and 40°. In the conventional petrolatum-based ointments the products are very soft and almost fluid at high summer heat and very firm at winter temperatures. The same ointment in a temperature-stable base such as Plastibase maintains a consistent degree of softness practically independent of temperature. This undoubtedly helps in improving consumer acceptance of the product.

HANDLING AND MANUFACTURING CHARACTERISTICS

The retail pharmacist usually compounds ointment prescriptions in one to two ounce quantities and seldom over a pound. In some manufacturing operations, however, ointments are often compounded in lots of 1,000 lb. and greater. This involves the use of large scale equipment and inasmuch as ointments are usually made by the batch process the equipment is often in use for a number of days for each batch.

An examination of many ointment formulas reveals that the fusion process is practically always involved in the preparation of an ointment in large scale manufacturing. These heating and cooling cycles are time consuming, tie up valuable equipment, and delay production.

The appearance and the texture of most ointments are usually improved considerably by milling. By the very nature of this process, production is usually slow and even with the large roller mills, yields of a few hundred pounds per hour are normal.

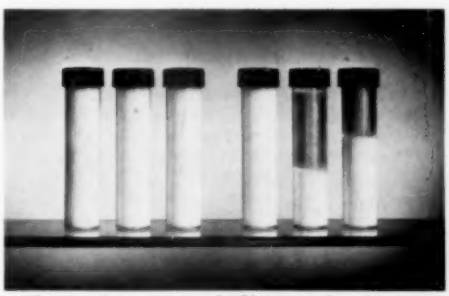


Fig. 9.—Appearance of 5% ammoniated mercury ointments stored overnight. Group of three at left were stored at room temperature. Group at right were stored at 48°. In both groups the bases are, from left to right: Plastibase, white petrolatum, U. S. P. and petrolatum-cholesterol.

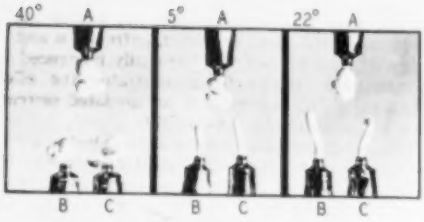


Fig. 10.—20% zinc oxide ointments in Plastibase (A), petrolatum-cholesterol (B), and petrolatum (C) at 5°, room temperature, and 40°.

The development of ointment formulas for large scale manufacturing must therefore take these production factors into consideration, and without sacrificing quality, provide for efficient manufacturing operations.

In our evaluation of ointment bases it became apparent to us that Plastibase possessed certain properties which might provide for an efficient manufacturing of ointments.

Conventionally, the addition of an active ingredient to an ointment base involves the fusion of the base, dispersion of the active ingredient in the fluid base, cooling, and finally, milling.

The softness and dispersion characteristics of Plastibase, together with its capacity for solids are such that ointments can be prepared with it by essentially the same procedure that is used by the retail pharmacist. Essentially this involves mixing the dry powder with a small portion of the base, levigating, and diluting. By mechanizing this process, ointments can be prepared in a much shorter period of time than by the fusion process.

This can perhaps best be illustrated by describing the manufacture of a 10,000 unit/gram penicillin ointment in Plastibase. Such an ointment would contain approximately 1% or less of solids, depending on the potency of the penicillin and on the amount of overage and other adjuncts.

Because Plastibase has a large capacity for solids, it is possible to make a 25% dispersion of solids in the base. This concentrate can be made very simply by mixing the powder in the base in a bowl equipped with a rotary beater. No heat is involved. The mixture is still very soft and actually not much more viscid than the base itself. The penicillin ointment concentrate in Plastibase can also be levigated or milled at this point. This means a considerable saving in milling time, because in this case only 1/25 of the volume of the finished product must be milled. The milled concentrate is transferred to another mixing bowl with rotary beater and the concentrate diluted with Plastibase to the required potency. This dilution is made by geometric progression with only a few minutes of stirring necessary between each addition of Plastibase. This produces a homogeneous dispersion.

Although Plastibase ointments are of soft ointment consistency, in large quantities they usually have flow properties. Because of this, transfers to storage tanks or to hoppers on filling machines can be made by hoisting the container and merely pouring into the selected receptacle. This overcomes the tedious and expensive hand transfer.

SUMMARY

A dialysis method and two extraction methods have been described and by these methods the drug-release characteristics of a number of bases have been compared. By these methods, Plastibase, a hydrocarbon gel of unique structure, is shown to possess release characteristics superior to the conventional hydrocarbon bases. There is evidence to suggest that these *in vitro* methods are indicative of *in vivo* activity.

The ease of spreadability of an ointment and the pressure required to extrude an ointment from a tube are presented as factors dependent on consistency but which lend themselves to a more realistic evaluation. Methods for measuring these factors are described. When tested by these methods, Plastibase is shown to be much softer than the conventional hydrocarbon bases.

At least one indication of the versatility of an ointment base can be obtained by determining its capacity for solids. These measurements have been made in terms of tube extrusion pressure and can also be expressed in poises.

The relative merits of various bases are discussed from the point of view of stability and manufacturing efficiency. A process is described for making penicillin ointment in Plastibase.

This paper is presented in the hope that it may stimulate the development of even more realistic evaluations of ointment bases in order to make the selection of an ointment base a truly rational process.

REFERENCES

(1) Jurist, A. E., *J. Investig. Dermatol.*, **20**, 331(1953).
(2) Blank, H., personal communication.
(3) Foster, S., Wurster, D. E., Higuchi, T., and Busse, L. W., THIS JOURNAL, **40**, 123(1951).
(4) Mutimer, M. N., Riffkin, C., Hill, J. A., Cyr, G. N., **45**, 101(1956).
(5) Robinson, R. C. V., *Arch. Dermatol. Syphilol.*, **72**, 54 (1955).
(6) Skauen, D. M., Cyr, G. N., Christian, J. E., Lee, C. O., THIS JOURNAL, **38**, 618(1949).
(7) Campbell, J. A., *ibid.*, **44**, 598 (1955).

1-Phenyl-2-Amino-3-Methoxy-1-Propanol Hydrochloride and Related Compounds*

By JEKISHAN R. PARIKH and J. F. ONETO

A method has been described for the preparation of 1-phenyl-2-amino-3-methoxy-1-propanol hydrochloride. It has been shown that the behavior of β-methoxypropiophenone toward nitrosation is influenced by the reaction solvent. α-Oximino-β-chloropropiophenone reacts with diethylamine and morpholine to form the corresponding β-substituted-amino α-oximinopropiophenones.

THE PREPARATION OF DL-*threo*- and DL-*erythro*-1-*p*-nitrophenyl-1-hydroxy-2-dichloroacetamido-3-methoxypropane diastereoisomers, as analogs of chloramphenicol has been reported by Rebstock (1). Prior to the appearance of this report in the literature, it occurred to us that the preparation of these analogs might possibly be achieved through the application of known series of reactions to 1-phenyl-2-amino-3-methoxy-1-propanol (I).

The main purpose of the investigation herein described was to explore procedures leading to the preparation of (I). A singular approach to this end consisted in the nitrosation of β-methoxypropiophenone, followed by catalytic hydrogenation.

β-Methoxypropiophenone was prepared according to a modified method of Leslie and Henze (2). Attempts to prepare β-methoxypropiophenone by Friedel-Craft's reaction on β-methoxypropionyl chloride and by Grignard or phenyllithium reactions on β-methoxypropionitrile did not materialize. When β-methoxypropionyl chloride was subjected to Friedel-Craft's reaction at 0 – 5° in the presence of anhydrous aluminum chloride and dry benzene, employing dry carbon disulfide as solvent, an appreciable amount of acrylophenone was obtained.

A number of modified procedures were investigated for the preparation of α-oximino-β-methoxypropiophenone (II), in which reaction conditions involving solvent, catalyst and temperature were varied. When the nitrosation of β-methoxypropiophenone was conducted according to the method of Hartung and Crossley (3), a nondistillable, brown, oily residue was isolated. With appropriate reagents the oily material afforded products characterized as di-2,4-dinitrophenylhydrazone and glyoxime derivatives of 1-phenyl-3-methoxy-1,2-propanedione. The expected 2,4-dinitrophenlyhydrazone of (II) was not obtained.

On subjecting the partially purified oily residue to catalytic reduction, employing 10 per cent palladium-on-charcoal in acidic medium (4), (I) was obtained as the hydrochloride in low yield. The ultraviolet absorption spectrum of the product was found to be in close agreement with the characteristic phenyl spectrum of 1-phenyl-2-amino-1-propanol hydrochloride rather than with the much stronger spectrum observed for α-aminopropiophenone hydrochloride, indicating the absence of a phenyl conjugated carbonyl function.

Referring to the considerable body of information available on the catalytic reduction of α-oximinoketones, Chang and Hartung (5) have indicated that the resulting amino alcohols occur as single racemic modifications of the *erythro* series. It may be assumed, therefore, by analogy, that (I) is the racemate conforming to the *erythro* configuration.

Utilizing methyl alcohol as the principal solvent, the nitrosation of β-methoxypropiophenone produced an oily product which also afforded the (I) hydrochloride when subjected to catalytic hydrogenation in acidic medium (4). Changing the conditions of the catalytic reduction procedure to conform to the method recently described by Hartung and Chang (6), in which palladium-on-charcoal is employed in the presence of alkaline medium, 1-phenyl-2-amino-1-propanol was isolated as the hydrochloride in poor yield.

It was of interest to find that while nitrosation of propiophenone in methanol in the presence of trace concentrations of hydrogen chloride proceeded smoothly to yield α-oximinopropiophenone in almost comparable yield to the one reported by Hartung and Crossley (3) employing a large excess of hydrogen chloride, the nitrosation of β-methoxypropiophenone did not materialize under similar conditions.

Nitrosation of β-methoxypropiophenone using alkaline catalysis (7,8) was not found to be

* Received September 16, 1955 from University of California, School of Pharmacy, San Francisco 22.

This paper represents an abstract of a dissertation submitted by Jekishan R. Parikh to the Graduate Division, University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

applicable on account of the instability of β-methoxypropiophenone in basic medium.

When the nitrosation of β-methoxypropiophenone was carried out following the procedure of Bruckner *et al.* (9), but employing benzene as the principal solvent, α-oximino-β-chloropropiophenone (III) was obtained. The identity of (III) was confirmed by comparison with an authentic product resulting from the direct nitrosation of β-chloropropiophenone.

Catalytic reduction of (III), employing palladium-on-charcoal in the presence of ethanolic hydrogen chloride, as well as reduction with stannous chloride, yielded α-aminopropiophenone hydrochloride in both instances.

Although (III) reacts with diethylamine and morpholine to give the corresponding α-oximino-β-tertiaryamino derivatives of propiophenone, it failed to yield (II) when reacted with sodium methoxide. This failure may possibly be attributed to the instability of (III) in the presence of strong base as evidenced by the decomposition of (III) in 5 per cent sodium hydroxide solution, yielding benzoic acid.

Attempts to prepare (I) by application of the Dakin and West reaction (10) to β-methoxyalanine as well as by direct methylation of α-nitro-β-hydroxypropiophenone using dimethyl sulfate failed.

Another approach to the synthesis of (I) consisted in the effort to methylate α-benzamido-β-hydroxypropiophenone. On conducting the reaction with dimethyl sulfate and sodium hydroxide at room temperature, the starting ketone was recovered unchanged. By increasing the reaction temperature to 40 – 50°, a product was isolated from the reaction mixture which proved to be 1,5-diphenyl-2,4-dibenzamido-1,5-pentadione (11). Subjecting the *p*-toluenesulfonic acid ester of α-benzamido-β-hydroxypropiophenone to the action of sodium methoxide failed to yield the corresponding alkyl ether.

EXPERIMENTAL[1]

β-Methoxypropiophenone.—The method employed was a modification of the one described by Leslie and Henze (2). The phenylmagnesium bromide was prepared in the usual way in a three-neck flask equipped with a mercury sealed Hirshberg stirrer, a dropping funnel, and a condenser using 21.6 Gm. (0.9 mole) of clean dry magnesium, 600 cc. of anhydrous ether, and a solution of 141.3 Gm. (0.9 mole) of dry bromobenzene in 150 cc. of dry ether. The mixture was cooled in an ice bath and 85.5 Gm. (0.46 mole) of anhydrous cadmium chloride was added in portions over a period of twenty minutes with vigorous stirring. The ice bath was then replaced by a hot water bath and the mixture refluxed for one and one-half hours. At the end of this time the Gilman test for phenylmagnesium bromide was negative. The mixture was cooled in an ice-salt bath and a solution of 91.5 Gm. (0.75 mole) of β-methoxypropionyl chloride in 450 cc. of dry benzene was added as rapidly as possible with vigorous stirring. It is essential that the condenser be cooled with circulating ice water, since considerable heat is evolved on addition of the acid chloride. Vigorous stirring must be maintained in order to break up the heavy sludge which is formed during the reaction. The ice bath was removed and the reaction mixture was stirred for an additional one and one-half hours. The reaction mixture was allowed to stand overnight and subsequently treated with hydrochloric acid-ice water mixture. The organic layer was separated and the aqueous layer extracted with ether. The organic layer was combined with the ether extracts and washed first with water, then with sodium bicarbonate solution, and again with water and finally dried over anhydrous calcium chloride. The solvents were removed at the water pump and the residue distilled under vacuum. The ketone distilled at 98–100° at 1–1.5 mm. pressure; yield, 84%; m. p. of its 2,4-dinitrophenylhydrazone, 175–176°. Leslie and Henze reported: yield, 90%; m. p. of 2,4-dinitrophenylhydrazone 175.5–176.5°(2).

Nitrosation of β-Methoxypropiophenone.—(*A*) *Procedure of Hartung and Crossley* (3). The reaction was carried out using 82 Gm. (0.5 mole) of β-methoxypropiophenone, 51.6 Gm. (0.5 mole) of *n*-butylnitrite and 350 cc. of anhydrous ether. After overnight standing at room temperature, 3.8 Gm. of unidentified crystalline material, m. p. 112–113°, was removed by filtration and the filtrate extracted repeatedly with cold 10% sodium hydroxide solution. The combined extracts were poured with stirring into a mixture of concentrated hydrochloric acid and crushed ice. A dark brown oil precipitated. The acid mixture was extracted with ether, the combined extracts washed with water and dried over anhydrous sodium sulfate. Removal of ether by vacuum distillation at room temperature left a dark brown oily residue weighing 50 Gm. Seventeen grams of unreacted ketone was recovered from the original ether reaction phase.

When a portion of the oily product was subjected to vacuum distillation at 1 mm. pressure, decomposition occurred. On steam distillation of a second portion of the product from 10% sulfuric acid, benzoic acid and benzaldehyde were recovered from the distillate.

Glyoxime of 1-Phenyl-3-methoxy-1,2-propanedione.—Oximation of 1 Gm. of the oily product from (*A*) using 1 Gm. of hydroxylamine hydrochloride, 5 cc. of dry pyridine, and 5 cc. of anhydrous ethanol gave 0.15 Gm. of crystalline product after recrystallization from methanol-water; m. p. 188–189°.

Anal.—Calcd. for $C_{10}H_{12}N_2O_3$: N, 13.45. Found: N, 13.33.

Di-(2,4-dinitrophenylhydrazone) of 1-Phenyl-3-methoxy-1,2-propanedione.—A 2,4-dinitrophenylhydrazone derivative prepared from the oily product

[1] All melting points are uncorrected. Microanalyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

obtained from (*A*) melted at 258–259°, after several recrystallizations from hot nitrobenzene to which hot ethanol was added to turbidity.

Anal.—Calcd. for $C_{22}H_{18}N_6O_9$: N, 20.92. Found: N, 21.05.

Hydrochloride of 1-Phenyl-2-amino-3-methoxy-1-propanol (I).—Following the general procedure of Hartung and Munch (4), 10 Gm. of the oily product from (*A*) (equivalent to 0.06 mole of α-oximino-β-methoxypropiophenone assuming the oil to represent the pure α-oximino ketone) was dissolved in 150 cc. of anhydrous ethanol containing 7 Gm. of hydrogen chloride. The mixture was hydrogenated in the presence of 5 Gm. of palladium-on-charcoal at 30 p. s. i. The crude reduction product weighing 7 Gm. was dissolved in hot anhydrous alcohol, treated with decolorizing carbon and filtered. The hot filtrate was mixed with an equal volume of dry benzene followed by the addition of dry ether to turbidity. Crystalline material precipitated on storing the mixture overnight in the icebox; m. p. 135–145°; yield, 3.5 Gm. An analytical sample was prepared by two additional recrystallizations from the same solvents; m. p. 144–147°.

Anal.—Calcd. for $C_{10}H_{16}NO_2Cl$: C, 55.16; H, 7.40; N, 6.43. Found: C, 55.26; H, 7.38; N, 6.23.

The ultraviolet absorption spectra of 1-phenyl-2-amino-1-propanol hydrochloride and 1-phenyl-2-amino-3-methoxy-1-propanol hydrochloride measured with a Cary spectrophotometer showed ϵ_{max} = 194.7 and ϵ_{max} = 206.8, respectively, at 257 mμ, while α-aminopropiophenone hydrochloride showed an ϵ_{max} = 12624 at 250 mμ in water.

***B*. Using Methanol as the Principal Solvent.**—The nitrosation reaction was carried out employing 32.8 Gm. (0.2 mole) of β-methoxypropiophenone, 36 Gm. of 20% ethereal hydrogen chloride (0.2 mole of hydrogen chloride) and 22.7 Gm. (0.22 mole) of isobutylnitrite in 400 cc. of dry methanol. The ethereal hydrogen chloride solution was added in one portion to a solution of the ketone in methyl alcohol. The nitrous ester was then added dropwise to the stirred mixture at room temperature. After standing for twelve hours the reaction mixture was treated with an excess of powdered calcium carbonate and extracted with ether. Removal of ether from one-half of the ether extract yielded 17.9 Gm. of oily residue. The residue was dissolved in ether, extracted with several small portions of 5% sodium bicarbonate solution and then with cold 1% sodium hydroxide solution. The cooled sodium hydroxide extract was saturated with carbon dioxide. An oily precipitate formed. The mixture was extracted with ether, the ether extract washed with water and dried over anhydrous sodium sulfate. Removal of the ether gave a pale yellow oily residue.

***p*-Nitrobenzoate of α-Oximino-β-methoxypropiophenone.**—Three grams of oily product obtained from *B* was treated with 3 Gm. of *p*-nitrobenzoylchloride in the presence of 15 cc. of dry pyridine. The crude product was recrystallized from ethanol, using decolorizing carbon; m. p. 95–98°; yield, 3 Gm. An analytical sample, m. p. 105–106°, was prepared by several additional recrystallizations from ethanol.

Anal.—Calcd. for $C_{17}H_{15}N_3O_8$: C, 59.64; H, 4.23; N, 8.18. Found: C, 59.79; H, 4.04; N, 8.22.

A portion of the oily product from (*B*) weighing 16.4 Gm. was reduced in 3 *N* absolute ethanolic hydrogen chloride using 5 Gm. of 10% palladium-on-charcoal. The resulting 10 Gm. of crude product was purified as described under (*A*); m. p. 145–148°; yield, 5.5 Gm. A mixed m. p. with the hydrochloride of (I) obtained under (*A*) showed no depression.

1-Phenyl-2-amino-1-propanol Hydrochloride.—Five grams of oily product from *B* was dissolved in 27 cc. of 5% absolute ethanolic sodium hydroxide and subjected to catalytic hydrogenation in the presence of 0.52 Gm. of palladium-on-charcoal, following the procedure of Hartung and Chang (6). A purified product prepared from the reduction mixture weighed 0.78 Gm., m. p. 189–191° (lit. 191°) (4).

The above procedure *B* was modified as follows using only a trace amount of hydrogen chloride: To a solution of 41 Gm. (0.25 mole) of β-methoxypropiophenone in 250 cc. of anhydrous methanol maintained at 10–15° was added a small portion of 25.8 Gm. (0.25 mole) of isobutyl nitrite followed by the addition of 2 cc. of 20% ethereal hydrogen chloride. The remainder of the nitrous ester was added over a period of forty minutes. After standing overnight at room temperature and removing the solvent under vacuum, 48.5 Gm. of oily residue remained. Vacuum distillation of 10 Gm. of the residue yielded 7 Gm. of unreacted β-methoxypropiophenone, identified through its 2,4-dinitrophenylhydrazone derivative. No other indentifiable products were isolated.

Applying the modified procedure to 13.42 Gm. (0.1 mole) of propiophenone, 15.47 Gm. (0.15 mole) of isobutylnitrite, 1.5 cc. of 20% ethereal hydrogen chloride and 100 cc. of anhydrous methanol gave the normally expected α-oximino-propiophenone; yield, 12 Gm.; m. p. 113–114.5°; (lit. 112–113°) (3).

***C*. Using Benzene as the Principal Solvent.**—The procedure of Bruckner *et al.* (9) was applied to 65.6 Gm. (0.4 mole) of β-methoxypropiophenone, 75.2 Gm. of 20% ethereal hydrogen chloride (0.42 mole hydrogen chloride) and 47.4 Gm. (0.44 mole) of isobutylnitrite in 400 cc. of anhydrous benzene. During the dropwise addition of the nitrous ester, the temperature of the reaction mixture rose to 40°. After standing at room temperature for twelve hours, 3 Gm. of crystalline inorganic material was removed by filtration and the filtrate was treated with powdered calcium carbonate. Filtration and subsequent vacuum distillation of the solvent resulted in a partially crystallized oily residue. The crystalline α-oximino-β-chloropropiophenone (III) was removed by filtration and washed with small portions of cold benzene; yield, 15 Gm.; m. p. 106–108°. An additional 3 Gm. of the crystalline product as well as 8 Gm. of unreacted β-methoxypropiophenone was recovered from the filtrate. An analytical sample was prepared by recrystallization first from 50% ethanol and then twice from ligroin; m. p. 108–109°.

Anal.—Calcd. for $C_9H_8NO_2Cl$: N, 7.09; Cl, 17.94. Found: N, 6.81; Cl, 18.05.

Nitrosation of β-Chloropropiophenone.—The nitrosation was carried out using 31.7 Gm. (0.2 mole) of β-chloropropiophenone, 37.6 Gm. of 20% ethereal hydrogen chloride (0.21 mole hydrogen chloride), 23.7 Gm. (0.22 mole) of isobutylnitrite and 200 cc. of anhydrous benzene. The reaction was con-

ducted at room temperature following essentially the procedure of Bruckner *et al.* (9). The yield of crude product was 25.4 Gm.; m. p. 105–107°. A mixed m. p. of the purified product with (III) obtained from the oximation procedure (*C*) showed no depression.

Glyoxime of 1-Phenyl-3-chloro-1,2-propanedione.—The derivative was prepared from 4 Gm. (0.02 mole) of (III) dissolved in 25 cc. of 95% ethanol and 2.8 Gm. of hydroxylamine hydrochloride dissolved in 25 cc. of water, following the procedure described by Levin and Hartung (12) for the oximation of phenylglyoxylohydroxamyl chloride. The crude product was recrystallized from ethanol-water and from toluene-ligroin combinations; m. p. 173–173.5°.

Anal.—Calcd. for $C_9H_9N_2O_2Cl$: N, 13.17. Found: N, 12.69.

When treated with sodium methoxide in anhydrous methanol, (III) decomposed to products which were not identified.

Reduction of α-Oximino-β-chloropropiophenone (III).—A solution of 11.8 Gm. (0.06 mole) of (III) dissolved in 150 cc. of anhydrous ethanol containing 7 Gm. of hydrogen chloride was hydrogenated at 15 p. s. i. in the presence of 5 Gm. of 10% palladium-on-charcoal. Six grams of crude product, m. p. 163–165° was isolated from the reduction mixture. An analytical sample was prepared by several recrystallizations from an alcohol-benzene-ether system, m. p. 178–180°.

Anal.—Calcd. for $C_9H_{12}NOCl$: N, 7.54. Found: N, 7.72.

A mixed m. p. determination of the product with an authentic sample of α-aminopropiophenone hydrochloride prepared by the method of Edkins and Linnell (13) melting at 179–181° (reported m. p. 187°) showed no depression. Hartung (14), who obtained the compound from the catalytic reduction of α-oximinopropiophenone, reported a m. p. of 183.5° (corr.).

The reduction of (III), using 8.63 Gm. (0.043 mole) of the ketone, 57 Gm. of anhydrous stannous chloride and 305 cc. of anhydrous ether, following the procedure of Edkins and Linnell (13) yielded 2.1 Gm. of crystalline product after recrystallization from an alcohol-benzene-ether system. An analytical sample was prepared by three additional recrystallizations from the same solvent system; m. p. 185–187°. The product analyzed for α-aminopropiophenone hydrochloride.

Anal.—Calcd. for $C_9H_{12}NOCl$: N, 7.54; Cl, 19.10. Found: N, 7.46; Cl, 19.18.

Ultraviolet absorption spectra observations for products from both the catalytic and stannous chloride procedures indicated the presence of phenyl conjugated carbonyl function.

α-Oximino-β-morpholinylpropiophenone Hydrochloride.—To a solution of 5.2 Gm. (0.059 mole) of dry morpholine in 30 cc. of anhydrous ethanol was added 4 Gm. (0.02 mole) of (III) in small portions over a period of fifteen minutes with adequate stirring. The temperature of the reaction mixture was maintained below 30°. The mixture, containing crystalline material which precipitated toward the end of the addition, was stirred for an additional fifteen minutes and then diluted with 185 cc. of anhydrous ether. The crystalline precipitate was filtered off and dried; yield 2.2 Gm.; m. p. 125–135°. After several recrystallizations from anhydrous alcohol, the product, identified as morpholine hydrochloride, melted at 171°. The ethereal filtrate was washed with water, dried over anhydrous magnesium sulfate and the ether removed to give a viscous oily residue. The residue was dissolved in ether, and treated with dry hydrogen chloride. Two and one-half grams of hygroscopic material, m. p. 140–146° with decomposition, was obtained after recrystallization from absolute ethanol. An analytical sample was prepared by three additional recrystallizations from the same solvent; m. p. 182–182.5°.

Anal.—Calcd. for $C_{13}H_{17}N_2O_3Cl$: N, 9.83. Found: N, 9.71.

α-Oximino-β-diethylaminopropiophenone Hydrochloride.—The compound was prepared from 4 Gm. (0.12 mole) of (III), 4.4 Gm. (0.06 mole) of dry diethylamine and 45 cc. of anhydrous ether following the above procedure. Recrystallization of the crude hydrochloride from absolute alcohol gave a crystalline product; m. p. 150–155°; yield, 1.5 Gm. An analytical sample was prepared by two additional recrystallizations from the same solvent, m. p. 154–155°.

Anal.—Calcd. for $C_{13}H_{19}N_2O_2Cl$: N, 10.34. Found: N, 10.72.

α-Nitro-β-hydroxypropiophenone.[2]—Nineteen Gm. (0.15 mole) of powdered sodium carbonate was added with stirring to a solution of 25 Gm. (0.15 mole) of α-nitroacetophenone (11) in 250 cc. of 95% ethanol followed by the dropwise addition of 12.5 cc. of a 37% solution of formalin (0.15 mole). After addition of the formalin solution, the stirring was continued for 45 minutes. Two hundred cc. of 20% acetic acid was added to the mixture and the resulting crystalline precipitate removed by filtration; yield, 25 Gm.; m. p. 126–130°. An analytical sample was prepared by two additional recrystallizations from an alcohol-acetone mixture; m. p. 150–151°.

Anal.—Calcd. for $C_9H_9NO_4$: N, 7.18. Found: N, 7.32.

The attempted methylation of the product in dioxane with dimethyl sulfate following the usual procedure failed to yield β-methoxy-α-nitropropiophenone.

Attempted Methylation of α-Benzamido-β-hydroxypropiophenone.—α-Benzamido-β-hydroxypropiophenone was prepared following the method of Long and Troutman (11). However, when the amounts of reactants were increased by a factor of 4, 26.5 Gm. of material melting at 208–209° after recrystallization from alcohol was obtained. The product was identified as 1,5-diphenyl-2,4-dibenzamido-1,5-pentadione by a mixed m. p. determination with an authentic sample prepared by the method of Long and Troutman (11). Attempted methylation of α-benzamido-β-hydroxypropiophenone was carried out in a manner similar to the procedure described by Gordon (15), using 10 Gm. (0.037 mole) of α-benzamido-β-hydroxypropiophenone in 170 cc. of dioxane, 1 *N* sodium hydroxide and 4 cc. of dimethyl sulfate. During the alternate addition of the base and methylating agent and for three additional hours, the temperature of the reaction mix-

[2] The authors wish to thank J. Goyan for his assistance in the preparation of this compound.

ture was maintained at 40–50°. The solvent was removed under vacuum. The oily residue solidified on treatment with 200 cc. of cold water; yield, 9.5 Gm.; m. p. 178–179.5°. An analytical sample was prepared by two recrystallizations from 50% alcohol, m. p. 209°; mixed m. p. with an authentic sample indicated the product to be 1,5-diphenyl-2,4-dibenzamido-1,5-pentadione.

Anal.—Calcd. for $C_{31}H_{26}N_2O_4$: C, 75.90; H, 5.34. Found: C, 75.68; H, 5.24.

Toluene-*p*-sulfonate of α-Benzamido-β-hydroxypropiophenone.—A mixture of 4.0 Gm. (0.015 mole) of α-benzamido-β-hydroxypropiophenone and 5 cc. of dry pyridine was cooled to 10°. To the cold suspension was added 3.1 Gm. (0.016 mole) of toluene-*p*-sulfonylchloride in divided portions over a period of twenty minutes. The resulting crystalline suspension was stirred for two hours, maintaining the temperature between 10–20°. The reaction mixture was treated with a mixture of 9 cc. of concentrated hydrochloric acid and 30 cc. of ice water. The crystalline product was removed by filtration, washed with 5% sodium bicarbonate solution, and then with water, and dried; yield, 4.6 Gm.; m. p. 108–110°. An analytical sample was prepared by recrystallization from 95% ethanol; m. p. 174–175°. The product analyzed for the monohydrate.

Anal.—Calcd. for $C_{23}H_{21}NO_5S \cdot H_2O$: C, 62.57; H, 5.25; S, 7.26. Found: C, 62.82; H, 5.42, S, 7.03.

Treatment of the tosyl ester in absolute methanol with sodium methoxide did not yield the expected α-benzamido-β-methoxypropiophenone.

REFERENCES

(1) Rebstock, M. C., *J. Org. Chem.*, **19**, 851(1954).
(2) Leslie, R. E., and Henze, H. R., *J. Am. Chem. Soc.*, **71**, 3480(1949).
(3) Hartung, W. H., and Crossley, F., "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, New York, 1943, p. 363.
(4) Hartung, W. H., and Munch, J. C., *J. Am. Chem. Soc.*, **51**, 2262(1929).
(5) Chang, Y., and Hartung, W. H., *ibid.*, **75**, 80(1953).
(6) Hartung, W. H., and Chang, Y., *ibid.*, **74**, 5927(1952).
(7) Claisen, L., and Manasse, O., *Ber.*, **20**, 2194(1887).
(8) Litvan, F., and Robinson, R., *J. Chem. Soc.*, 1997 (1938).
(9) Bruckner, V., Fodor, G., Kiss, J., and Kovacs, J., *ibid.*, 888(1948).
(10) Dakin, H. D., and West, R. J., *J. Biol. Chem.*, **78**, 91, 745, 757(1928).
(11) Long, L. M., and Troutman, H. D., *J. Am. Chem. Soc.*, **71**, 2469(1949).
(12) Levin, N., and Hartung, W. H., *J. Org. Chem.*, **7**, 408(1942).
(13) Edkins, R. P., and Linnell, W. H., *Quart. J. Pharm. and Pharmacol.*, **9**, 75(1936).
(14) Hartung, W. H., *J. Am. Chem. Soc.*, **53**, 2248(1931).
(15) Gordon, A. H., *Biochem. J.* (London), **37**, 538(1943).

Anticoagulant Sulfuric Acid Esters of Synthetic Polyglucoses*

By DALE P. J. GOLDSMITH, KANE L. KELLEY, and CHARLES W. MUSHETT

The anticoagulant activity and toxicity of a series of sulfated synthetic polyglucoses were studied. The samples possessed 10–40 per cent the activity of heparin and were 2.5–10 times as toxic as heparin. The results obtained are similar to those reported for other sulfated polysaccharides.

THE ANTICOAGULANT ACTIVITY of sulfuric acid esters of polysaccharides is well known (1). Heparin is the most important member of this class of compounds and many different carbohydrates have been sulfated in an effort to find a substitute for it. These synthetic preparations, however, have proved to be too toxic for clinical use. Recently synthetic polyglucoses prepared by the acid polymerization of glucose have become available (2, 3). The novelty of these polysaccharides prompted the current study on the anticoagulant activity and toxicity of their sulfated derivatives.

EXPERIMENTAL

Polyglucose Sulfuric Acid Esters.—A series of polyglucose fractions (Fractions 1–7, Table I) of different degrees of viscosity were prepared from several batches of acid polymerized glucose.[1] These were converted, respectively, to the sulfate esters (Fractions A–G, Table I) by heating in a mixture of pyridine (40 cc./Gm. of polyglucose) and chlorosulfonic acid (4.4 cc./Gm. of polyglucose) at 65–75° for 5 hours. Following reaction, the mixture was cooled and the pyridine layer was decanted. The residue was dissolved in water, the solution was made alkaline with sodium hydroxide, and the resulting sodium salt of sulfated polyglucose was precipitated with alcohol. This sodium salt was reprecipitated from water with alcohol and then dialyzed in water at about *p*H 7 for several days, after which the sac content was lyophilized.

* Received September 9, 1955, from the Chemical Division of Merck and Company, Inc., and the Merck Institute for Therapeutic Research, Rahway, New Jersey.

[1] Kindly supplied by Dr. Arthur J. Zambito, Developmental Research Department, Chemical Division, Merck and Company, Inc., Rahway, New Jersey.

TABLE I.—PROPERTIES OF POLYGLUCOSE FRACTIONS AND CORRESPONDING SULFATED DERIVATIVES

Polyglucose Starting Material		Sulfated Polyglucose					
Fraction	Intrinsic Viscosity	Fraction	Recovery[a]	S,[b] %	Na,[b] %	Act.,[c] u./mg.	I.V. LD_{50} Mice, mg./Kg.
1	0.54	A	1.9	19.0	13.0	35	175
2	0.36	B	1.2	..	..	30	330
3	0.28	C	1.4	14.1	9.8	40	460
4	0.20	D	1.1	13.3	10.5	13	380
5	0.14	E	1.2	12.7	8.5	45	280
6	0.11	F	0.9	14.6	9.8	55	250
7	0.08	G	0.6	18.7	12.7	50	175
		F-1	..	16.2	12.0	55	185
		F-2	..	7.3	5.6	13	700
		F-3	..	1.5	..	1	>1500

[a] Grams of sulfated polysaccharide per Gm. of polyglucose.
[b] 12.1% S and 8.7% Na is equivalent to one $-SO_3Na$ group per saccharide unit; 17.5% S and 12.6% Na is equivalent to two such groups per saccharide unit.
[c] The standard heparin employed contained 130 u./mg. and had an LD_{50} of 1750 mg./Kg.

In addition, Fraction F, Table I, was precipitated from sodium chloride solution by successive additions of ethanol to give Fractions F-1, F-2 and F-3. The recoveries were 59, 36 and 4 per cent, respectively.

Electrophoretic patterns were run on two representative fractions, namely Fractions C and F, at 0.5 per cent concentration in 0.1 *M* phosphate buffer of *p*H 7. Each of these exhibited three boundaries with anionic mobilities of 5.0×10^{-5}, 9.8×10^{-5}, 11.2×10^{-5}, and 3.8×10^{-5}, 10.9×10^{-5}, 11.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, respectively.

Other pertinent data on the polyglucose fractions and their sulfated derivatives are given in Table I.

Activity and Toxicity.—The sulfated polysaccharides were assayed *in vitro* for anticoagulant activity by the "solution assay" method described previously (4) with heparin as the reference standard. Acute intravenous toxicity was determined on the Harpaul strain of white mice. The results obtained are shown in Table I. With the exception of Fraction F-3, which contained an insignificant amount of sulfur, the anticoagulant activity of the sulfate esters varied from 10–40 per cent that of heparin and the toxicity from 2.5–10 times that of heparin. It is of interest to note that sulfated Fraction D which had an LD_{50} of 380 mg./Kg. was prepared from a polyglucose fraction which was completely nontoxic at 3700 mg./Kg.

The efficacy of Fraction D was studied in the rabbit following intravenous administration of 5 mg./Kg. The coagulation time of a mixture of 0.5 ml. whole blood plus 0.5 ml. physiological saline was determined at 37.5° at various intervals after injection of the heparinoid. The data obtained were as follows (hours after injection—clotting time in minutes): 0–2.2, 0.5–35.5, 1–17.3, 2–11.3, 3–10.8, 4–7.8, 5–1.8. It is evident that this sample effected an elevation in coagulation time which persisted for at least four hours and returned to normal by the fifth hour.

DISCUSSION

The sulfated polyglucose fractions prepared in this work were found to be strongly anticoagulant, with 10–40 per cent the activity of heparin *in vitro*. One sample, administered intravenously to the rabbit, effected decreased coagulability of the blood for at least 4 hours. Toxicity, in general, increased as the amount of sulfur in the derivatives increased, but activity appeared to show no consistent relationship to the sulfur content. Neither the efficacy nor the toxicity of the sulfated derivatives exhibited a direct relationship to the viscosity of the polyglucose starting materials. This may be due in part to the polydisperse nature of the initial polyglucose fractions and the selective separation of the higher molecular weight components upon sulfation. Evidence for this latter assumption is suggested by the decrease in yield of ester with decrease in viscosity of polyglucose.

The toxicity of other sulfated polysaccharides has been shown to be related to their size, shape and molecular weight (5) as well as to the structure and solubility of the parent carbohydrate (6). The toxicity of the synthetic polyglucose sulfuric acid esters is probably related to similar factors. In particular, it would appear from the toxicity data on an unsulfated polyglucose sample that such starting material did not contribute by hydrolysis or contamination to the toxicity observed for the final sulfated products.

The results obtained in this study are in general similar to those reported for the sulfation of other polysaccharides. The synthetic polyglucoses, therefore, would not appear to offer any easier approach to a useful heparin substitute than do other carbohydrates. Work towards such a heparinoid using synthetic polyglucose as a starting material would require exacting control of both initial polysaccharide and sulfated derivative such as is necessary with other carbohydrates.

SUMMARY

A series of synthetic polyglucoses of varying viscosity have been converted to their sulfate esters and the latter have been studied as heparin-like anticoagulants. The better samples had 10–40 per cent the anticoagulant activity of heparin and were 2.5 to 10 times as toxic as heparin. The problems involved in the preparation of a clinically useful anticoagulant from the synthetic polyglucoses appear about the same as those involved with other polysaccharides.

REFERENCES

(1) Walton, K. W., *Brit. Med. Bull.*, **11**, 62(1955).
(2) Pacsu, E., and Mora, P. T., *J. Am. Chem. Soc.*, **72**, 1045(1950).
(3) Heidelberger, M., and Aisenberg, A. C., *Proc. Natl. Acad. Sci. U.S.*, **39**, 453(1953).
(4) Mushett, C. W., Goldsmith, D. P. J., and Kelley, K. L., *J. Biol. Chem.*, **211**, 163(1954).
(5) Walton, K. W., *Brit. J. Pharmacol.*, **7**, 370(1952).
(6) Karrer, P., Koenig, H., and Usteri, E., *Helv. Chim. Acta*, **26**, 1296(1943).

A Pharmacologic Study of Combinations of Antihistaminic Substances*

By JOSEPH A. KAISER† and CASIMIR T. ICHNIOWSKI

A preliminary pharmacologic study of some antihistaminic combinations has been conducted. Sixty-two mixtures prepared from ten compounds were screened for their antiasthmatic activity. Because of their high antiasthmatic activity eleven combinations were selected for further study. Two binary mixtures, both containing Pyribenzamine® Hydrochloride and Thephorin® Tartrate, were found to possess a supra-additive antihistaminic activity, which may offer certain clinical advantages. The acute toxicity of the mixtures, as determined in the guinea pig was shown to be only additive in effect.

THE ART of combining drugs for the attainment of certain specific therapeutic benefits is very old indeed. Only recently, however, have the histamine antagonists been combined with one another or with other medicaments. Such mixtures may possess a wider spectrum of activity, reduced toxicity, increased potency, fewer side effects and longer duration of activity.

In 1952 Simon and Toohey (1) reported that a combination of Pyribenzamine® hydrochloride and Antistine® hydrochloride, each in one-half the usual dose, was effective in the treatment of various allergic states. They reported further that this therapy was almost totally free of untoward responses. Similarly, beneficial results from the use of combinations of antihistamine agents have been reported by Hubbard and Berger (2), Wittich (3), and Bonvini and Grasso (4). The earliest laboratory report noted was that of Naranjo and Naranjo (5). In their study of twenty-five combinations, a ternary combination of 40 per cent Neo-Antergan® maleate, 30 per cent Trimeton® maleate and 30 per cent Neohetramine® hydrochloride proved to be the most active. The antiasthmatic combinatory efficiency index of this combination was reported to be 2.38.

In view of the fact that there might be some therapeutic advantage in the use of combinations of antihistamine agents, a preliminary laboratory investigation was conducted with hopes of securing a mixture which might possess certain unique features, clinically desirable.

EXPERIMENTAL

Materials.—The following ten histamine antagonists were used in the preparation of sixty-two mixtures: Bromodiphenhydramine hydrochloride (Ambodryl® hydrochloride, Parke, Davis), diphenhydramine hydrochloride (Benadryl® hydrochloride, Parke, Davis), methapyrilene hydrochloride and methapyrilene fumarate (Chemo Puro Manufacturing Corporation), phenindamine tartrate (Thephorin® Tartrate, Hoffmann-LaRoche), prophenpyridamine maleate (Trimeton® maleate, Schering), pyrilamine maleate (Neo-Antergan® maleate, Merck), pyrrobutamine diphosphate (Pyronil® diphosphate, Eli Lilly), thonzylamine hydrochloride (Neohetramine® hydrochloride, Nepera), tripelennamine hydrochloride (Pyribenzamine® hydrochloride, Ciba). Histamine phosphate U. S. P. was the histamine salt used.

Anti-asthmatic Activity.—The procedure of Siegmund, Granger, and Lands (6) was used to determine the antiasthmatic activity of the histamine antagonists. Each guinea pig was subjected to a pretest by being placed in an atmosphere of a finely dispersed histamine mist. Compressed air maintained at a pressure of 300 mm. of mercury was used for the nebulization of a 0.2% aqueous solution of histamine phosphate contained in a DeVilbiss

* Received July 25, 1955, from College of Pharmacy, University of Maryland, Baltimore.
Abstracted in part from a thesis submitted by Joseph A. Kaiser to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Present Address: Pfizer Therapeutic Institute, Maywood, N. J.
The authors wish to extend sincere thanks to the following who have donated so generously many of the compounds used in this study: Dr. R. H. Barry of Schering, Dr. J. Cooper of Ciba Pharmaceutical Products, Dr. A. H. Fiske of Eli Lilly and Co., Dr. P. Gottfried of Merck, and Dr. G. W. Mast of Nepera Chemical Company.

TABLE I.—ANTIASTHMATIC STUDIES OF SINGLE ANTIHISTAMINE AGENTS

Drug	Code[a]	No. G. P.	Protective Dose$_{50}$ 95% Confidence Limits Lower–Upper mg./Kg.	Relative Potency
Methapyrilene hydrochloride	MH	80	0.35 0.24–0.52	1,229
Methapyrilene fumarate	MF	58	0.80 0.57–1.13	538
Ambodryl® hydrochloride	A	60	1.18 0.76–1.83	364
Thephorin® tartrate	T	60	1.57 1.22–2.03	274
Pyribenzamine® hydrochloride	P	70	1.70 1.23–2.34	253
Benadryl® hydrochloride	B	60	4.30 2.87–6.45	100

[a] Abbreviations (below) are used when necessary in succeeding tables.

No. 40 nebulizer. Animals not succumbing to convulsions within a period of two minutes were not used. After a rest of at least three hours, the animals were given an intraperitoneal injection of the antihistaminic dissolved in distilled water. Thirty minutes later they were again placed in the histamine atmosphere. All animals re-exposed to the mist for at least six minutes without showing symptoms of histamine intoxication were considered protected. The animals were rested five days or more before being subjected to this routine again.

Antispasmodic Activity.—An isolated tissue procedure utilizing the guinea pig ileum served in evaluating the antispasmodic activity. The method was essentially that reported by Chen, Ensor, and Clarke (7).

The experimental design involved the determination of the amount of antihistaminic preparation which would suppress the spasmogenic effect of a submaximal dose of histamine. A suppressive dose was rated "positive" if it antagonized the spasm by 75% or more and "negative" if the degree of antagonism were less than 75%. This method of "all or none" scoring was adopted from the procedure of Miller, Becker, and Tainter (8). The dose of the spasmogen which produced suitable submaximal effects, was 0.2 μg. histamine base/ml. of fluid bathing the tissue.

Blood Pressure Studies.—The procedure of Graham (9) was followed in the determination of the protection afforded by the antihistaminics against histamine-induced vasodepression. Cats under phenobarbital sodium anesthesia were used. The degree of inhibition of histamine vasodepression was expressed in terms of per cent of the control histaminic responses.

Acute Toxicity Study.—The acute toxicities of

TABLE II.—ANTIASTHMATIC STUDIES OF ANTIHISTAMINIC COMBINATIONS

No.	Composition	Protective Dose$_{50}$ 95% Confidence Limits Lower-Upper mg./Kg.	Probable Protective Dose Effect of Component Fraction Dose: mg./Kg.	% Protection	Index of Combinatory Efficiency 95% Confidence Limits Lower-Upper	Relative Potency	Number of animals
C-10	2/3 P	0.37	0.25	3.0	4.47	1162	60
	1/3 T	0.34–0.41	0.12	0.0	4.03–4.87		
C-8	1/2 P	0.41	0.205	1.8	3.98	1049	40
	1/2 T	0.29–0.56	0.205	0.05	2.91–5.63		
C-29	4/5 A	0.36	0.29	4.6	2.99	1194	60
	1/5 MF	0.19–0.68	0.07	0.6	1.58–5.67		
C-13	1/3 P	0.46	0.15	0.8	2.87	935	60
	2/3 A	0.33–0.62	0.31	5.5	2.13–4.00		
C-36	1/3 P	0.34	0.113	0.4	2.16	1265	70
	1/3 T	0.29–0.40	0.113	0.0	1.83–2.54		
	1/3 MH		0.113	18.5			
C-3	2/3 P	1.05	0.70	19.0	2.03	410	30
	1/3 B	0.58–1.91	0.35	1.4	1.11–3.67		
C-49	1/3 T	0.57	0.19	0.04	1.92	754	60
	1/3 A	0.37–0.88	0.19	1.6	1.25–2.96		
	1/3 MF		0.19	7.0			
C-22	1/5 T	0.53	0.106	0.0	1.80	811	60
	1/5 A	0.35–0.80	0.106	0.25	1.20–2.73		
	3/5 MF		0.318	17.0			
C-55	1/4 T	0.58	0.145	0.0	1.73	741	60
	1/4 A	0.42–0.80	0.145	0.65	1.26–2.39		
	1/2 MF		0.29	3.8			
C-2	1/3 P	2.0	0.67	17.0	1.42	215	40
	2/3 B	1.33–3.02	1.33	15.5	0.94–2.14		
C-1	1/2 P	1.82	0.91	27.0	1.34	236	30
	1/2 B	1.39–2.38	0.91	10.0	1.03–1.76		

TABLE III.—ANTISPASMODIC STUDIES OF SINGLE ANTIHISTAMINE AGENTS

Drug	No. Trials	Effective Dose$_{50}$ 95% Confidence Limits Lower-Upper μg./ml.	Relative Potency
Methapyrilene hydrochloride	44	0.0061 (0.0051–0.0073)	1,043
Methapyrilene fumarate	46	0.0107 (0.0080–0.0142)	594
Pyribenzamine® hydrochloride	114	0.0176 (0.0143–0.0216)	361
Ambodryl® hydrochloride	72	0.0324 (0.0277–0.0379)	196
Thephorin® tartrate	70	0.0605 (0.0442–0.0829)	105
Benadryl® hydrochloride	145	0.0636 (0.0553–0.0731)	100

the histamine antagonists and their combinations were determined in guinea pigs deprived of food for 24 hours. Administrations were made intraperitoneally. At least forty animals were used for each drug. As a rule a four-dose level design was employed.

RESULTS AND DISCUSSION

All of the experimental data were analyzed statistically by the method of Litchfield and Wilcoxon (10). Tests for parallelism indicate that the regression lines are parallel within a 95% confidence limit. Benadryl® hydrochloride was employed as the standard for comparison in all the experimental procedures. The results are presented in tabular form, Tables I to VI. In addition to the usual parameters of activity, the combinatory efficiency index (5) was calculated for each combination in the following manner: C. E. I. = fP/cP in which fP is the relative potency established experimentally, and cP the calculated potency for the combination on the assumption of additive [iso addition, Loewe (11)] behavior of the components. $cP = qP_1 + rP_2 + vP_3 \ldots$ in which 1, 2, and 3 refer respectively to the components, P is the potency relative to Benadryl® hydrochloride, and q, r, and v are the respective proportions of the components. When the C. E. I. = 1, it indicates additive behavior, while C. E. I. less than 1 and C. E. I. greater than 1, indicate respectively infra- and supra-additive effects.

Of a total of sixty-two combinations screened for anti-asthmatic activity, eleven were of sufficient activity to warrant further testing. The results obtained with these eleven preparations are summarized in Tables II and IV.

TABLE IV.—ANTISPASMODIC STUDIES OF ANTIHISTAMINIC COMBINATIONS

No.	Composition	Effective Dose$_{50}$ 95% Confidence Limits Lower-Upper μg./ml.	Probable Antispasmodic Dose Effect of Component Fraction Dose: μg./ml.	% Response	Index of Combinatory Efficiency 95% Confidence Limits Lower-Upper	Relative Potency	Number of Trials
C-8	1/2 P	0.0093	0.00315	0.3	4.33	1,010	40
	1/2 T	(0.0047–0.0084)	0.00315	0.012	(3.25–5.81)		
C-10	2/3 P	0.0060	0.0040	0.8	3.84	1060	42
	1/3 T	(0.0044–0.0082)	0.0020	0.0	(2.81–5.24)		
C-13	1/3 P	0.0113	0.0038	0.7	2.24	563	36
	2/3 A	(0.0090–0.0141)	0.0075	0.1	(1.80–2.82)		
C-36	1/3 P	0.0077	0.00256	0.1	1.64	826	48
	1/3 T	(0.0062–0.0096)	0.00256	0.0	(1.32–2.04)		
	1/3 MH		0.00256	0.3			
C-29	4/5 A	0.0156	0.0124	2.0	1.48	408	56
	1/5 MF	(0.0126–0.0193)	0.0031	2.2	(1.20–1.83)		
C-49	1/3 T	0.0154	0.00513	0.13	1.38	413	38
	1/3 A	(0.0122–0.0194)	0.00513	0.0	(1.10–1.74)		
	1/3 MF		0.00513	11.5			
C-1	1/2 P	0.0200	0.01	18.0	1.38	318	76
	1/2 B	(0.0142–0.0282)	0.01	0.1	(0.98–1.94)		
C-3	2/3 P	0.0202	0.01347	34.0	1.15	315	46
	1/3 B	(0.0146–0.0279)	0.00673	0.0	(0.83–1.59)		
C-22	1/5 A	0.0140	0.0028	0.0	1.09	454	36
	1/5 T	(0.0100–0.0203)	0.0028	0.0	(0.75–1.53)		
	3/5 MF		0.0084	35.0			
C-2	1/3 P	0.0360	0.012	27.0	0.94	177	80
	2/3 B	(0.0288–0.0450)	0.024	5.0	(0.75–1.18)		
C-55	1/4 T	0.0208	0.0052	0.1	0.82	306	36
	1/4 A	(0.0156–0.0277)	0.0052	0.0	(0.62–1.09)		
	1/2 MF		0.0104	47.0			

TABLE V.—INHIBITION OF HISTAMINE VASODEPRESSION

Drug	Effective Dose[a] 95% Confidence Limits Lower-Upper mg./Kg.	Probable Inhibition Dose Effect of Component Fraction Dose: mg./Kg.	% Inhibition	Index of Combinatory Efficiency 95% Confidence Limits Lower-Upper	Relative Potency	Number of Responses
Pyribenzamine® hydrochloride	0.35 0.12–0.97				280	22
Thephorin® tartrate	0.47 0.16–1.40				208	24
C-8 1/2 P 1/2 T	0.55 0.16–1.93	0.275 0.275	45.5 39.0	0.72 0.21–2.52	178	17
C-10 2/3 P 1/3 T	0.68 0.23–2.04	0.453 0.227	56.0 34.7	0.55 0.18–1.67	144	20
Benadryl® hydrochloride	0.98 0.32–2.96				100	27

TABLE VI.—ACUTE TOXICITY

Drug	Lethal Dose[a] 95% Confidence Limits Lower-Upper mg./Kg.	Probable Lethal Dose Effect of Component Fraction Dose: mg./Kg.	% Response	Index of Combinatory Efficiency 95% Confidence Limits Lower-Upper	Relative Toxicity	Number of Animals
Pyribenzamine® hydrochloride	71 44.4–113.6				137	55
C-10 2/3 P 1/3 T	82 70.1–96.0	54.7 27.3	42.0 0.0	0.997 0.86–1.17	119	55
C-8 1/2 P 1/2 T	88 67.7–114.4	44.0 44.0	29.0 0.1	1.004 0.77–1.30	111	40
Benadryl® hydrochloride	97.5 87.1–109.2				100	40
Thephorin® tartrate	116 98.3–136.9				84	49

An analysis of the anti-asthmatic data obtained with C-8 is interesting. C-8 is composed of 50% Pyribenzamine® hydrochloride and 50% Thephorin® tartrate. The protective dose of the combination for 50% of the animals ($P.D._{50}$) is 0.41 mg./Kg.; of Pyribenzamine® hydrochloride, 1.70 mg./Kg.; of Thephorin® tartrate 1.57 mg./Kg. The dose-effect relationships for these three drugs show that a dose of 0.41 mg./Kg. of Pyribenzamine® hydrochloride would be expected to protect only 8% of the injected guinea pigs. At one-half of this dose, namely 0.205 mg./Kg., approximately 1.8% of the animals so treated could be expected to be protected. A similar picture presents itself when dealing with Thephorin® tartrate. A dose of 0.41 mg./Kg. would be expected to protect only 1.5% of the animals and if only 0.205 mg./Kg. were administered, probably all of the animals would succumb to the effects of the histamine. The activity of the two drugs in C-8 is difficult to explain.

In the test of the antispasmodic activity of the eleven combinations as tested on the isolated guinea pig ileum, combinations C-8 and C-10 proved to be the most efficient. The combinatory efficiency index as of C-8 and C-10 are 4.3 and 3.8 respectively.

The test employed to determine the ability of the antihistaminics to antagonize histamine vasodepression in the cat was not sufficiently reliable to render accurate data. For this reason only the two most efficient combinations were tested. Results reported are to be considered only as approximations of degree of activity.

The toxicologic efficiency ratings of C-8 and C-10, for practical purposes, may be considered to be unity. The increase in antagonistic action of the combinations to histamine with only an additive toxicity may be indicative of a better therapeutic potential.

REFERENCES

(1) Simon, S. W., and Toohey, J. T., *Ann. Allergy*, **10**, 484(1952).
(2) Hubbard, T. F., and Berger, A. J., *ibid.*, **8**, 350(1950).
(3) Wittich, F. W., *ibid.*, **10**, 625(1952).
(4) Bonvini, E., and Grasso, E., *Med. Press*, London, **228**, 602(1952).
(5) Naranjo, P., and Naranjo, E. B., *Ann. Allergy*, **11**, 699(1953).
(6) Siegmund, O. H., Granger, H. R., and Lands, A. M., *J. Pharmacol. Exptl. Therap.*, **90**, 254(1947).
(7) Chen, G., Ensor, C. R., and Clarke, I. G., *ibid.*, **92**, 90(1948).
(8) Miller, L. C., Becker, T. J., and Tainter, M. L., *ibid.*, **92**, 260(1948).
(9) Graham, J. D. P., *ibid.*, **91**, 103(1947).
(10) Litchfield, J. T., Jr., and Wilcoxon, F., *ibid.*, **96**, 99 (1949).
(11) Loewe, S., *ibid.*, **106**, 404(1952).

A Paper Chromatographic Study of Aloe, Aloin and of Cascara Sagrada*

By NOURI Y. MARY,† BERNARD V. CHRISTENSEN, and JACK L. BEAL

A qualitative and quantitative study of aloe, aloin, and cascara sagrada by the use of paper chromatography was undertaken. The results indicated the presence of aloe-emodin and anthranol in the free state and in glycosidal combination in all five of the drugs investigated. In addition, the Curaçao aloe contained chrysophanic acid and the cascara sagrada contained chrysophanic acid and emodin in their free state and also in glycosidal combination.

A LITERATURE REVIEW of aloe indicated that there is still considerable question with regard to its chemical composition. In addition, it was found that the work that appeared in the literature was conducted almost entirely on one official species of aloe (Curaçao). This investigation was undertaken to ascertain the major constituents present in the three official species of aloe, and to quantitate, if possible, the active constituents present, utilizing the technique of paper chromatography.

Since aloin is prepared from aloe a study of a sample of aloin was included in this investigation. Cascara sagrada having similar type major constituents was also included in the investigation with the hope that paper chromatography would shed new light on the constituents. As far as the authors could determine, paper chromatography has never been employed in the analysis of these drugs. Paper chromatography, however, has been applied by Shibata and Takido (1) for the qualitative and quantitative studies of several varieties of rhubarb.

QUALITATIVE STUDIES

Preparation of Authentic Specimens of Some Anthracene Derivatives.—The nature of the investigation undertaken necessitated that pure samples of some of the anthracene derivatives be available. Accordingly, the following compounds were prepared: aloe-emodin, aloe-emodin anthranol, chrysophanic acid, and emodin.

Aloe-emodin was prepared according to the method of Cahn and Simonsen (4). Aloe-emodin anthranol was prepared according to Hauser's method (5). Chrysophanic acid and emodin were prepared by the processes of John H. Gardner (6, 7). The pure compounds were identified by their melting points and by a study of their absorption curves.

Spectrophotometric Examination of the Prepared Anthracene Derivatives.—The maximum absorption of a substance is an important criterion for its identification. It also provides a means of quantitating the substance under investigation. Therefore, the transmittance curves of the pure anthracene derivatives prepared previously were determined. The results illustrated the points of maximum absorption of aloe-emodin and chrysophanic acid at 430 mμ, emodin at 440 mμ, and aloe-emodin anthranol at 360 mμ.

Identification of Constituents

(a) **Extraction.**—Chloroform extracts of the free and the combined (glycosidal combination) anthracene derivatives from verified powdered specimens of Cape aloe, Socotrine aloe, Curaçao aloe, aloin, and cascara sagrada were prepared by Daels' method (8) as modified by Brody, Voigt and Maher (2), using smaller volumes of the extracting menstruum (25 ml. chloroform per 5 Gm. of drug).

(b) **Paper chromatography.**—The method used for the analysis of the drug extracts was basically that of Shibata and Takido (1). Several strips of chromatographic paper, Whatman No. 1, 60 cm. long and 4 cm. wide, were prepared. The test solutions in chloroform containing the free and combined anthracene derivatives prepared above were spotted with a micro-pipet on a start line which was drawn at a 4-cm. distance from one of the ends of each of the strips. Volumes of the chloroform solutions ranging from 100–250 λ were employed. The strips were then allowed to air-dry before chromatographing them.

The chromatography was carried out by the ascending method using petroleum ether (b. p. 65–110°) saturated with 97 per cent methyl alcohol at room temperature. The bottom of a chromatographic jar (30 cm. in diameter and 60 cm. in length) was covered to a depth of two inches with the above solvent. The chamber was closed tightly and left for about 6 hours to ensure atmospheric saturation.

The strips containing the test solution were stapled on a grid made of five glass rods. The ends of the strips bearing the test solution were the farthest from the rods. A maximum of 20 paper strips could be hung on the grid at each chromatographic run. The strips were transferred to the solvent-saturated chromatographic chamber which was then completely sealed to maintain the atmospheric equilibrium. The chromatograms were allowed to develop for 10-12 hours.

The strips were removed from the chromatographic jar and the solvent fronts marked. After drying, the developed paper strips were sprayed with 0.5 per cent methanolic magnesium acetate solution; the strips then were heated at 100° for

* Received September 7, 1955, from College of Pharmacy, The Ohio State University, Columbus 10.
An abstract of a part of a dissertation presented to the Graduate School of the Ohio State University by Nouri Y. Mary in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Present address: Royal College of Pharmacy and Chemistry, Baghdad, Iraq.

5 minutes resulting in a distinct orange, pink or yellow coloration.

Paper strips on which were spotted the pure authentic anthracene derivatives previously prepared were chromatographed simultaneously with the strips containing the test solution to indicate the exact R_f values. In addition, the comparison of the transmittance curves of the alcohol-eluted compounds from the unknown chromatograms with the transmittance curves of the compounds eluted from the known chromatograms served as a further criterion for identification.

Table I illustrates the R_f values of the spots that appeared and the color they gave with the methanolic magnesium acetate solution. Table II contains the qualitative results of the anthracene derivatives found in the samples studied.

TABLE I.—R_f VALUES OF ANTHRACENE COMPOUNDS AND THE COLOR OF THESE CIMPOUNDS WITH THE MAGNESIUM ACETATE REAGENT

Compound	R_f Value	Color of Compound with Methanolic Magnesium Acetate Reagent
Anthranol	0.09	Yellow
Aloe-emodin	0.25	Orange, becoming pink
Emodin	0.44	Orange, becoming pink
Chrysophanic acid	0.94	Orange, becoming pink

TABLE II.—ANTHRACENE DERIVATIVES FOUND IN THE SAMPLES STUDIED

Anthracene Derivative	Curaçao aloe	Cape aloe	Socotrine aloe	Aloin	Cascara sagrada
Anthranol	+	+	+	+	+
Aloe-emodin	+	+	+	+	+
Chrysophanic acid	+	−	−	−	+
Emodin	−	−	−	−	+

DISCUSSION

As indicated in Table II the experiments conducted on Curaçao aloe revealed the presence of anthranol, aloe-emodin, and chrysophanic acid in both the free state and in glycosidal combination. The anthranol was identified on the basis that it conformed to previously reported characteristics in that: (*a*) it exhibited no absorption peak at any wavelength over a wide range of the ultraviolet and visible portions of the spectrum; (*b*) it gave a yellow color with alkalies instead of the orange or pinkish coloration characteristic of the other anthracine derivatives. This yellow coloration was also noticed by Brody, Voigt and Maher (2) and Gibson and Schwarting (3) who employed an alkaline chromatographic column for separating the anthracene derivatives of Curaçao aloe and cascara sagrada, respectively. These results of the Curaçao aloe differ from that of Brody, Voigt and Maher (2) in that chrysophanic acid was isolated and iso-emodin was not, while Brody, Voigt and Maher isolated iso-emodin and not chrysophanic acid.

In the analysis of cascara sagrada, the anthracene derivatives emodin, aloe-emodin, chrysophanic acid, and anthranol were detected as indicated in Table II. This differs from the results of Gibson and Schwarting (3) in that chrysophanic acid was detected and iso-emodin was not, while Gibson and Schwarting isolated iso-emodin and not chrysophanic acid. Le Prince (14) and Liddell, King and Beal (12, 15) have reported the presence of chrysophanic acid in cascara sagrada. Jowett (11), in addition to Gibson and Schwarting, did not report the presence of chrysophanic acid in cascara sagrada.

One might at first believe that these two compounds, iso-emodin and chrysophanic acid, have been confused. However, evidence of their different physical properties shows that not to be the case. In this experimental work the identity of chrysophanic acid was established by the following: First, it was soluble in ammonia giving a positive Borntrager reaction (16) (iso-emodin does not dissolve in ammonia and thus gives a negative Borntrager reaction). Second, it had the same absorption maximum as pure chrysophanic acid. Third, it gave an R_f value identical with the R_f value of a known pure sample of chrysophanic acid having a melting point of 196°. This R_f value agreed closely with the R_f value of chrysophanic acid prepared and chromatographed by Shibata and Takido (1). These two investigators found that anthraquinone compounds having two hydroxyl groups and one methyl group in their anthracene nucleus such as chrysophanic acid and its isomers usually yielded a very high R_f value (0.92). The introduction of a third hydroxyl group into the anthracene nucleus as in emodin and iso-emodin gave a much lower R_f value (0.50).

The presence of only anthranol and aloe-emodin was detected in the free state and in glycosidal combination in the Cape and Socotrine aloes. No chrysophanic acid could be detected on the chromatograms of their extracts. An unidentified constituent having a slightly higher R_f value than anthranol and an absorption maximum at 430 mμ was detected in the free state in Socotrine aloe. It was thought, at first, that some tailing of the aloe-emodin spot occurred, but repeated experiments under the same conditions revealed the separate existence of this extra spot.

The aloin sample used was found to contain only anthranol and aloe-emodin. The chemical composition of aloin differs according to its commercial source (17). Since no chrysophanic acid could be spotted on the chromatograms of its extracts, it was concluded that the aloin sample was not obtained from Curaçao aloe.

At no time in any of the drugs studied could aloe-emodin anthranol, first described by Hauser (5), be detected on the chromatograms. The pure aloe-emodin anthranol prepared according to Hauser's method (5) yielded two components when chromatographed. One of these components was aloe-emodin and the other component was identical with the anthranol present in all the drugs investigated here. It is possible, therefore, that the compound of Hauser is not a single entity but rather a two component substance.

QUANTITATIVE STUDIES

A comparative quantitative study of the official kinds of aloe was undertaken after separation of the constituents by the use of paper chromatography was achiev.d.

Moisture Determination.—The moisture content was determined by the U. S. P. XIV method (17) for drugs containing no volatile principles at 100°. The purpose of performing the moisture determination was to obtain data so that succeeding calculations could be made on a dry weight basis.

Determination of Water-Soluble Extractive.—The amount of water-soluble extractive was determined by the U. S. P. XIV method (17). The results appear in Table III.

TABLE III.—PERCENTAGE OF WATER-SOLUBLE EXTRACTIVE OF ALOE

Aloe	Per cent of Water-soluble extractive
Curaçao	80.89
Cape	70.04
Socotrine	41.95

Per cent Recovery of Anthracene Compounds from Chromatograms.—It has been reported in the literature that in quantitative practice, compounds separated by paper chromatography are not recovered completely from the developed chromatograms. Whether this was also true of the anthracene compounds was not known.

A 200 λ volume of a one per cent alcohol solution of each of aloe-emodin and chrysophanic acid was spotted on the starting line of a number of chromatographic strips and was chromatographed ascendingly for 10–12 hours. After drying, the strips were eluted with alcohol in a desiccator overnight. The eluents containing the anthracene compounds were diluted to a 10-ml. volume, their transmittance determined spectrophotometrically and the values interpreted from a calibration curve plotted previously on 2 x 10 semilogarithmic paper.

The results indicated that recovery was not complete. The average percentage recovery was 88.5 of aloe-emodin and 86.9 of chrysophanic acid. No satisfactory explanation has been reached which accounts for the loss on elution.

Quantitative Determination of the Measurable Anthracene Derivatives of Aloe.—Chloroform extracts of the anthracene derivatives of aloe present in the free state as well as in glycosidal combination were prepared separately by the method previously described. Depending on the color of the extracts, volumes ranging from 100–500 λ were spotted on paper strips and chromatographed ascendingly as previously described. After establishing the identity of the spots, they were eluted with ethyl alcohol, their transmittance read on a Beckman spectrophotometer, Model DU and their concentration determined from the standard curves previously plotted on 2 x 10 semilogarithmic paper taking into consideration the moisture content of the powdered drugs and the percentage of the compounds recovered on elution.

Only the anthracene compounds having an absorption maximum could be evaluated. These compounds included aloe-emodin and chrysophanic acid. The results of the analysis of all the aloes appear in Table IV.

The total anthracene content could not be determined since anthranol, a compound present in all aloes in the free as well as the combined state, could not be quantitatively determined by the same spectrophotometric method used for the determination of the other constituents, as no single wavelength could be chosen for its analytical determination.

TABLE IV.—QUANTITATIVE STUDIES OF ALOE

Sample	Curaçao aloe	Cape aloe	Socotrine aloe
Free Aloe-emodin expressed in mg. %	22.15	6.70	6.26
Combined Aloe-emodin expressed in mg. %[a]	149.73	60.12	37.62
Free Chrysophanic acid expressed in mg. %	7.84	...	...
Combined Chrysophanic acid expressed in mg. %[a]	33.77	...	...

[a] Combined refers to that occurring in glycosidal combination.

DISCUSSION

The results of the detemination of the water-soluble extractive (Table III) by the U. S. P. XIV method (17) showed that the Curaçao aloe contained more water-soluble extractive than the Cape aloe and Socotrine aloe. The difference in the amount of water-soluble extractives is not as great or significant as the difference in the amount of the anthracene derivatives. The data in Table IV show that there was considerable difference in the aloe-emodin content in the three varieties of aloe investigated. The Curaçao aloe was found to be the richest in the amount of aloe-emodin present. It contained approximately 2.5 times as much aloe-emodin as the Cape aloe and 4 times as much aloe-emodin as the Socotrine aloe. In addition, the Curaçao aloe contained a considerable amount of chrysophanic acid. It is concluded, therefore, that the Curaçao aloe was superior in quality to either the Cape or Socotrine aloes.

The data in Table IV reveal also that the free extracts of aloe contained far less anthracene compounds than the combined extracts. In the Curaçao aloe, approximately one-seventh of the aloe-emodin and one-fourth of the chrysophanic acid were found in the free state. In the Cape aloe one-ninth of the aloe-emodin occurred free and in the Socotrine aloe approximately one-sixth of the aloe-emodin was in the free state.

SUMMARY

1. The constituents of Curaçao aloe, Cape aloe, Socotrine aloe, aloin, and cascara sagrada were successfully separated by the technique of ascending paper chromatography, using Whatman No. 1 filter paper and petroleum ether (b. p. 65–110°) saturated with 97 per cent methyl alcohol as the solvent. The results indicated the presence of aloe-emodin and anthranol in the free state and in glycosidal combination in all five of the drugs investigated. In addition, the Curaçoa aloe contained chrysophanic acid and the cascara

sagrada contained chrysophanic acid and emodin in their free state and also in glycosidal combination.

2. Iso-emodin previously reported present in Curaçao aloe and cascara sagrada was not detected in either of these two drugs.

3. Paper chromatography was utilized in the quantitative evaluation of the three official species of aloe. The results showed the superiority of the Curaçao aloe to either the Cape or the Socotrine aloes.

REFERENCES

(1) Shibata, S., and Takido, M., *J. Pharm. Soc. Japan*, **72**, 2789(1950).
(2) Brody, T. M., Voigt, R. F., and Maher, F. T., THIS JOURNAL, **39**, 666(1950).
(3) Gibson, M. R., and Schwarting, A. E., *ibid.*, **37**, 206 (1948).
(4) Cahn, R. S., and Simonsen, J. L., *J. Chem. Soc.*, 2573 (1932).
(5) Hauser, F. *Pharm. Acta Helv.*, **6**, 79(1931).
(6) Gardner, J. H., THIS JOURNAL, **23**, 1178(1934).
(7) Gardner, J. H., *ibid.*, **28**, 143(1939).
(8) Daels, M. F., *Bull. acad. roy. méd. Belg.*, **29**, 779 (1929).
(9) Thorpe, T. E., and Miller, A. K., *J. Chem. Soc.*, **61**, 1(1892).
(10) Schwabe, P., *Arch. Pharm.*, **226**, 569(1888).
(11) Jowett, H. A. D., *Proc. Am. Pharm. Assoc.*, **52**, 288 (1904).
(12) Green, M. W., King, C. G., and Beal, G. D., THIS JOURNAL, **27**, 95(1938).
(13) Keimatsu, S., and Hirano, I., *J. Pharm. Soc. Japan*, **51**, 230(1931).
(14) Le Prince, M., *Compt. rend.*, **129**, 60(1899).
(15) Liddell, R. W., King, C. G., and Beal, G. D., THIS JOURNAL, **31**, 161(1942).
(16) Fairbairn, J. W., *Pharm. J.*, **148**, 198(1942).
(17) "The Pharmacopeia of the United States", 14th rev., Mack Publishing Co., Easton, Pa., 1950, pp. 26, 777, and 780.

A Simplified Assay of Nux Vomica Tincture*

By MORTON SCOTT†, ABRAHAM TAUB‡, and CLAUDE PIANTADOSI§

The time of the N. F. assay has been materially shortened by employing a preliminary isolation procedure involving adsorption on aluminum oxide followed by elution of the alkaloids with 70 per cent alcohol. The eluate is then subjected to ion exchange on Amberlite® IRC 50 and subsequent elution with 0.1 *N* HCl in 70 per cent alcohol. Final determination of strychnine and brucine, is made spectrophotometrically at 255 and 264 mμ. A batchwise ion exchange technique has been developed which has been found to be simpler and more rapid than the conventional columnar technique.

THE OFFICIAL ASSAY for strychnine in nux vomica galenicals is of good precision but relatively time consuming. The lengthy nature of the determination is due for the most part to difficulties encountered in the extraction of the strychnine and in the elimination of the brucine which always accompanies the strychnine in such preparations.

Numerous investigations have been conducted with the object of improving the official method of assay (1–3). Chromatographic procedures have been studied (4, 5) and the chromatographic separation of strychnine and brucine has been reported by La Rocca and Burlage (6) and others (7, 8). El Ridi and Khalifia (9) described a spectrophotometric assay which employed a preliminary purification of the alkaloids by adsorption of the galenical on alumina, followed by elution of the alkaloids with ethanol. Spectrophotometric methods for the simultaneous determination of strychnine and brucine have been reported (10, 11), and Bhattacharya and Ganguly (12) examined the deviations from Beer's law which may occur during such determinations. Analyses based on ion exchange techniques have also been described (13, 14). Recently, Piantadosi (15) suggested an improved assay based on the use of cation exchange resins. This procedure offered several advantages but the method still entailed the removal of brucine via oxidation followed by a tedious extraction of the strychnine.

In reviewing the official assay method and the work of others, it became apparent that a need still existed for a more rapid assay for strychnine in nux vomica galenicals. The present work describes such an assay for strychnine in nux vomica tincture, and it is believed that the method can be easily extended to include the assay of other nux vomica galenicals.

* Received May 5, 1955, from the Department of Chemistry, College of Pharmacy, Columbia University.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.
† Present address: Assistant in Chemical Engineering, Columbia University.
‡ Professor of Pharmaceutical Chemistry.
§ Present address: College of Pharmacy, Butler University, Indianapolis, Ind.

EXPERIMENTAL

In the preliminary studies an attempt was made to separate brucine and strychnine via ion exchange techniques. For these experiments Amberlite® IRC-50 (Rohm and Haas) in its sodium, hydrogen, and ammonium forms and under varying conditions of *p*H was employed. Since the ratio of *Kd* values (ion exchange distribution coefficient) for strychnine:brucine was nearly unity for all systems studied, the use of this method for separation purposes was of no value.

An attempt was then made to isolate the combined alkaloids directly from an assayed nux vomica tincture using Amberlite® IRC-50. It was found that complete adsorption of the alkaloids onto the hydrogen form of the resin occurred if the sample was adjusted to *p*H 5.25 or above. However, this resulted in precipitation of coloring principles and slight coprecipitation of the alkaloids. A preliminary purification was therefore found necessary.

Preliminary Purification of the Alkaloids.—On the basis of the experiments of Brownlee (5) and others (9, 16), a preliminary isolation of the alkaloids and removal of coloring principles was devised employing a batchwise treatment of the tincture with alumina. Satisfactory retention of color was obtained when 5 ml. of the tincture was stirred together with 5 Gm. of aluminum oxide (chromatographic grade, Merck). Selective elution of the alkaloids was accomplished with 70% alcohol. This batchwise elution of the adsorbed alkaloids was easily followed by spectrophotometric comparison of the eluates with the original sample and/or semiquantitative tests with Mayer's reagent. For quantitative recoveries of the alkaloids, it was found necessary to treat the alumina with at least 50 ml. of 70% alcohol (utilized in divided portions).

The subsequent adsorption of the alkaloids onto the Amberlite® IRC-50 was facilitated by making these eluates definitely alkaline (*p*H 8–10) by the addition of ammonia T.S.

Ion Exchange Studies.—Aliquots (25 ml.) of the eluates collected above were then passed through resin columns (2 cm., i.d.) containing 9–10 cm. (backwashed and drained) of acid-regenerated Amberlite® IRC-50. Flow rates were set at 1 ml. per minute. The effluents were collected and gave negative tests for alkaloids with Mayer's reagent.

Spectrophotometric examination of these solutions using a Beckman Model DU spectrophotometer and quartz cells indicated that the extraneous materials present in the alumina eluates were passing through the exchange column. The absorption spectrum of a typical effluent is presented in Fig. 1, curve 1. Also appearing in Fig. 1 is a plot of the absorption spectrum of the alumina eluate before passage through the resin bed (curve 2). The spectrum of the substances retained on the ion exchanger has been calculated by subtracting graphically curve 1 of Fig. 1 from curve 2. The resulting curve is shown as curve 3 of Fig. 1 and has been replotted as curve 1 of Fig. 2. For comparison, the absorption spectrum of a solution prepared to contain similar concentrations of the alkaloids is shown as curve 2 of Fig. 2. The close agreement of these curves indicated that the alkaloids adsorbed on the Amberlite® were in a relatively uncontaminated condition.

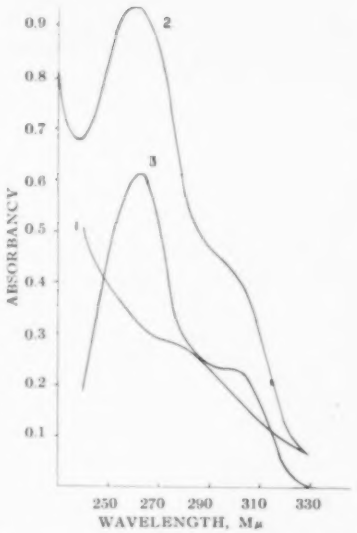


Fig. 1.—Absorption spectra of the fractions isolated during the purification of the alkaloids. (1) Effluent from the resin treatment of the alumina eluate. (2) Eluate from the alumina treatment of Nux Vomica Tincture. (3) Alkaloids adsorbed on the resin.

The batchwise ion exchange treatment of the alkaloids was studied by mixing solutions of strychnine and/or brucine in 70% alcohol with the acid-regenerated Amberlite® IRC-50. Contact period ranged from 5 minutes to 24 hours. The super-

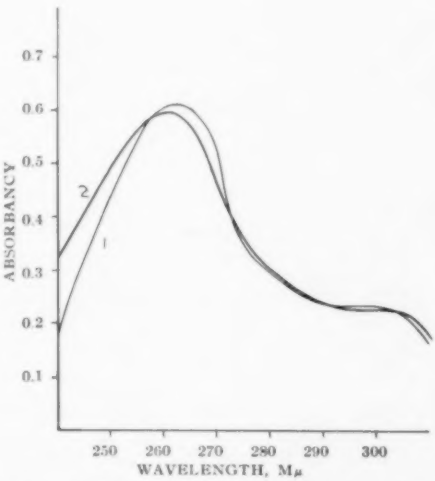


Fig. 2.—Comparison of the absorption spectrum of the alkaloids retained on Amberlite IRC-50 with that of a solution containing similar concentrations of brucine and strychnine in 70% alcohol. (1) Alkaloids retained on resin. (2) Prepared solution of brucine and strychnine.

natant liquids were collected and the resin rinsed with 70% alcohol. These washings were also collected. The resin containing the adsorbed alkaloid was then treated with the particular elutriant under study.

Quantitative data on the adsorption of alkaloids were obtained by calculation from the $E(1\%, 1\text{ cm.})$ values of the alkaloids in 70% alcohol and from the absorbance of the filtrates collected in the adsorption cycle. Spectrophotometric examinations were made at wavelengths of maximum absorbance (255, 264, and 302 mμ). Both alkaloids were found to obey Beer's law (in a concentration range up to 25×10^{-4} Gm. %) at these wavelengths. The E(1%, 1 cm.) values used in these ion exchange calculations may be obtained from Fig. 3 which presents the absorption spectra of strychnine and brucine in 70% alcohol.

The ability of the Amberlite® to retain alkaloid was examined under a variety of operating conditions including varying alkaloid concentrations, resin-sample contact time, degree of agitation, temperature, and volume ratio of sample to resin. Complete adsorption was accomplished when 10–20 ml. of the alkaloidal standards (containing 0.1–1.0 mg. total alkaloid per ml.) or 10–20 ml. of the alumina eluates collected in the previous steps was stirred for 15 minutes with 10 ml. of the resin. Stirring was necessary for complete adsorption. Temperature variation did not materially affect the adsorption process. The slight loss of alkaloid (estimated at 0.7%) could be made negligible by using very small quantities of 70% alcohol in the rinsing operations.

For the batchwise elution of the adsorbed alkaloids a series of elutriants including various concentrations of sulfuric acid, hydrochloric acid, and ammonium hydroxide in aqueous and alcoholic solutions was evaluated. The effects of resin-elutriant contact time, temperature, agitation, and volume ratio of elutriant to resin were also examined. The progress of the elution was followed by comparing the absorbance of the eluates (suitably diluted with water where necessary) with that of solutions containing known concentrations of brucine and strychnine. Complete recovery of the adsorbed alkaloid was achieved using 4 treatments of the resin with 25 ml. of 0.1 *N* hydrochloric acid in 70% alcohol. During each treatment the mixtures were stirred and warmed to 80° for 5 minutes. All other elutriants and elution procedures examined resulted in incomplete recoveries of the alkaloids.

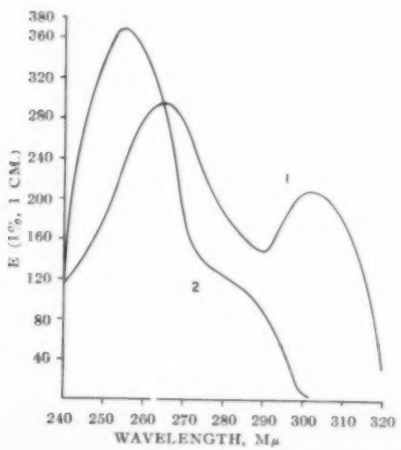


Fig. 3.—Absorption spectra for brucine and strychnine in 70% alcohol. (1) Brucine. (2) Strychnine.

On occasion, the eluates collected in these experiments exhibited a slight turbidity which interfered with the spectrophotometric determinations. To obtain solutions of absolute clarity, filtration through a Selas #010 porous filtering crucible was found necessary. It was also noted that the resin itself released soluble impurities which would interfere with the spectrophotometric measurements. The concentration of these substances in the final eluates was brought to a minimum value by carefully purifying and preconditioning the resin before use. The method of purification is described below. The importance of preparing sample blanks ("resin blanks") for all spectrophotometric measurements was nevertheless clearly established.

Spectrophotometric Studies.—For the spectrophotometric examination of solutions containing both alkaloids, samples were prepared to contain variable concentrations of brucine in a fixed concentration of strychnine, and vice versa. The solvent was 0.02 *N* hydrochloric acid in 14% alcohol. Duplicate measurements of absorbance were taken for each sample at the wavelength of maximum absorption for strychnine (255 mμ) and brucine (264 mμ). Figure 4 presents the results of these studies. That the absorbances of the alkaloids are additive at 264 and 255 mμ is indicated by the linear relationships shown in Fig. 4. It may also be noted that extrapolation of the curves back to zero concentration of the variable component gives rise to absorbance values for the fixed component which are identical with those determined experimentally. These results suggest, that in the present case, the effects of optical interference which may occur when both alkaloids are present in the same solution (12) are of a minor nature. Thus, the simultaneous spectrophotometric determination of the alkaloids should proceed without complication. The E (1%, 1 cm.) values to be used in such determinations have been calculated from the intercepts of Fig. 4 and are listed in Table I.

TABLE I.—E (1%, 1 CM.) VALUES FOR STRYCHNINE AND BRUCINE IN A SOLVENT OF 0.01 *N* HYDROCHLORIC ACID IN 14% ALCOHOL

	E (1%, 1 cm.)	
	255 mμ	264 mμ
Strychnine	346.9	289.9
Brucine	215.0	299.9

THE PROPOSED ASSAY PROCEDURE

Preparation of the Resin.—Approximately 100 Gm. of Amberlite® IRC-50 (H), analytical grade, is purified by steam distillation for three hours. About 200 ml. of 6 *N* sodium hydroxide is then added to the resin. The mixture is stirred occasionally within a 24-hour period, and the super-

natant liquid decanted. The treatment is repeated using 200 ml. of 6 *N* hydrochloric acid as regenerant. Purification of the resin is continued by repeating the base-acid cycles twice more.

At the end of the third acid treatment, 100 ml. of 3 *N* hydrochloric acid in 70% alcohol is added. The mixture is stirred occasionally during a 24-hour contact period. The excess acid is decanted and the resin is washed with deionized water until the last washings are free of chloride ion, as indicated by silver nitrate T.S. The resin is stored under 70% alcohol until ready for use.

Assay Procedure.—Ten grams of aluminum oxide (chromatographic grade, Merck) is placed in a 30-ml. beaker and a 5-ml. sample of nux vomica tincture is added followed by 5 ml. of 70% alcohol. A blank is prepared using 5 ml. of alcohol in place of the tincture.

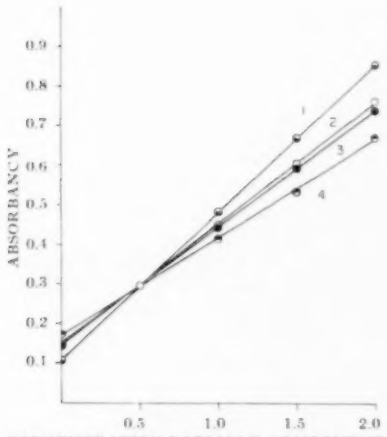


Fig. 4.—Absorbance of mixed solutions of strychnine and brucine at 255 mμ and 264 mμ (solvent—0.02 *N* HCl in 14% alcohol). (1) Strychnine in fixed concentration of brucine (0.50 mg./100 ml.) at 255 mμ. (2)Brucine in fixed concentration of strychnine (0.50 mg./100 ml.) at 264 mμ. (3) Strychnine in fixed concentration of brucine (0.50 mg./100 ml.) at 264 mμ. (4) Brucine in fixed concentration of strychnine (0.50 mg./100 ml.) at 255 mμ.

The alumina mixture is stirred for five minutes, the solid is allowed to settle, and the supernatant liquid is filtered through a medium-rapid filter paper into a 50-ml. volumetric flask. The alumina and the walls of the beaker are rinsed with small portions of 70% alcohol, filtering each fraction into the volumetric flask. The volume of liquid is brought to the mark by the addition of subsequent rinsings with 70% alcohol.

Ten milliliters of the eluate, adjusted to *p*H 9–10 with ammonia T.S., is added to 10 ml. of the prepared resin (previously washed with several small portions of 70% alcohol). The mixture is continuously stirred for 15 minutes, using a gentle motion with a polyethylene tipped glass rod, avoiding attrition of the resin. The resin is allowed to settle and the supernatant liquid is filtered avoiding excessive transfer of the resin particles to the filter paper. The resin is washed with two 10-ml. portions of 70% alcohol and these washings and the previous filtrate are discarded. The funnel and filter paper, with the traces of resin, are retained for the next step.

Twenty-five milliliters of 0.1 *N* hydrochloric acid in 70% alcohol is added to the resin and the mixture is cautiously heated on a steam bath to 80°. The resin is stirred gently for five minutes with a polyethylene tipped glass rod. The resin is then allowed to settle and the supernatant liquid is filtered through the filter paper and funnel arrangement retained above. The filtrate is collected in a 100-ml. volumetric flask and the procedure is repeated using three additional 25-ml. volumes of elutriant. The resin, the walls of the beaker and the filter paper are rinsed with a small portion of the elutriant and the solution is brought to the mark and mixed.

A 10-ml. aliquot is then diluted to 50 ml. in a volumetric flask, using distilled water. After mixing it is filtered with suction through a Selas No. 010 porous crucible. When approximately 10 ml. of filtrate is collected its absorbance is measured at 255 mμ and 264 mμ against a filtrate from a similarly treated resin blank.

The strychnine concentration is calculated by solving the following simultaneous equations:

$$A^{255m\mu} = (346.9)(S) + (215.0)(B)$$
$$A^{264m\mu} = (289.9)(S) + (299.9)(B)$$

where: S and B represent the concentrations of strychnine and brucine respectively, and $A^{255\ m\mu}$ and $A^{264\ m\mu}$ represent the absorbance of the sample at 255 mμ and 264 mμ, respectively.

The value obtained by solution of the above equations is multiplied by 500 to convert it to Gm. of strychnine per 100 ml. of tincture.

The time required for the assay is approximately 70 minutes, and multiple samples can be analyzed simultaneously at less than half this time per sample.

A comparison of the results obtained with the proposed assay and the N. F. assay is presented in Table II.

TABLE II.—STRYCHNINE CONCENTRATIONS IN NUX VOMICA TINCTURE AS CALCULATED BY THE PROPOSED ASSAY AND BY THE N. F. ASSAY

Proposed Assay Strychnine (mg. per 100 ml. of tincture)	N. F. Assay Strychnine (mg. per 100 ml. of tincture)
117.0	114.6
117.0	115.2
116.0	115.2
114.0	...
113.0	...
114.0	...
Mean 115.2	Mean 115.0

Samples containing specific increments of strychnine in two different tinctures of nux vomica were then prepared and analyzed by the proposed assay. Table III presents the comparative findings.

TABLE III.—RESULTS OBTAINED WITH INCREMENT SOLUTIONS

Sample Tr. Nux Vomica	N. F. Assay Av. Strychnine mg./100 ml.	Strychnine Increment mg./100 ml.	Strychnine by Proposed Assay mg./100 ml.	Percentage Difference
A	115.0	43.5	156.0	−1.6
A	115.0	43.5	159.0	+0.3
B	128.4	50.0	182.4	+2.2
B	128.4	50.0	182.0	+2.1
			Mean of % Differences	0.75

DISCUSSION

The results presented in Tables II and III indicate that the proposed assay compares favorably with the N. F. assay method. This new assay method has the advantage of requiring only 70 minutes for completion as opposed to the six to eight hours required by the official assay. In addition, the assay is relatively simple to perform and requires only a small sample volume (5 ml.).

CONCLUSIONS

1. A new assay method for strychnine in nux vomica tincture using simple ion exchange and spectrophotometric techniques has been developed.

2. The accuracy and precision of this new method compares favorably with that of the official method.

3. The new assay is more rapid than the N.F. procedure, and, in addition, can be performed on much smaller sample volumes.

REFERENCES

(1) Burlage, H. M., Jacobs, M. L., and Le Blanc, F. J., THIS JOURNAL, **22,** 298(1933).
(2) Horn, G. M., Haufman, K. L., and Mittelstaedt, S. G., *ibid.*, **29,** 183(1940).
(3) Allport, N., and Allen, J., *Quart. J. Pharm. and Pharmacol.*, **13,** 252(1940).
(4) Bjorling, J., *Svensk. Farm. Tids.*, **48,** 137(1944).
(5) Brownlee, G. W., *Quart. J. Pharm. and Pharmacol.*, **18,** 163(1945).
(6) La Rocca, J., and Burlage, H. M., *J. Elisha Mitchell Sci. Soc.*, **61,** 220(1945).
(7) Fisher, J., and Buchegger, B., *Pharm. Zentralhalle*, **89,** 147(1950).
(8) Jensen, von K. B., and Svendsen, H. B., *Pharm. Acta. Helv.*, **25,** 31(1950).
(9) El Ridi, M. S., and Khalifia, K., *J. Pharm. and Pharmacol.*, **4,** 191(1952).
(10) Jentzsch, K., *Scientia Pharm.*, **19,** 219(1951).
(11) Demoen, P., and Janssen, P., *J. pharm. Belg.*, **7,** 80(1952).
(12) Bhattacharya, R. N., and Ganguly, A. K., *J. Pharm. and Pharmacol.*, **4,** 485(1952).
(13) Jindra, A., *ibid.*, **1,** 87(1949).
(14) Jindra, A. and Pohorsky, J., *ibid.*, **3,** 344(1951).
(15) Piantadosi, C., "A Simplified Method of Analysis for Nux Vomica Galenicals," M. S. Thesis, College of Pharmacy of Columbia University, 1952.
(16) Brown, N. G., Kirch, E. R., and Webster, G. THIS JOURNAL, **37,** 24(1948).

The Toxic Component of the Singletary Pea (*Lathyrus pusillus*)*

By HAROLD P. DUPUY and JORDAN G. LEE

The lathyrogenic entity of the Singletary pea is γ-glutamyl-β-aminopropionitrile. The toxicity resides in the β-aminopropionitrile portion of the molecule. A qualitative test was developed which showed the presence of γ-glutamyl-β-aminopropionitrile and the absence of free β-aminopropionitrile in the seed of *Lathyrus odoratus* and *Lathyrus pusillus*. This test was negative for both compounds when applied to seed of the reportedly more toxic *L. latifolius*.

AN EARLIER PAPER from this laboratory (1) described a method for obtaining a toxic crystalline material from the seed of the Singletary pea (*Lathyrus pusillus*). Paper chromatographic examination of this material indicated that it was a mixture with one predominant substance. The three components were apparently closely related as they all yielded the same two amino acids on hydrolysis.

The mixture was separated on a column of powdered cellulose. Only the predominant material showed signs of toxicity when fed to weanling albino rats.

Analysis of the isolated predominant fraction, the toxic material, indicated a most probable formula of $C_8H_{12}N_2O_3$. One of the nitrogen

* Received July 7, 1955, from the Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge.

atoms was present in a readily hydrolyzed form identified as a nitrile group. A second nitrogen atom was present as an uncombined α-amino acid structure.

Paper chromatographic analyses and isolation of derivatives indicated that glutamic acid and β-alanine were present in the hydrolysates of the toxic material. Tagging with fluorodinitrobenzene showed definitely that only the amino group of glutamic acid was free in the toxic material.

At this stage of the investigation, the paper of Schilling and Strong (2) appeared which presented evidence that the toxin of *L. odoratus* was γ-glutamyl-β-aminopropionitrile. It was evident from the evidence cited above that the lathyrogenic compound of the Singletary pea is the same compound.

The β-aminopropionitrile portion of the molecule produced the classical symptoms of lathyrism in the rat as well as did the peptide. Of the related compounds fed, Table I, only aminoacetonitrile and bis(β-cyanoethyl)amine produced lathyritic symptoms. The aminoacetonitrile was extremely toxic, while the bis-(β-cyanoethyl)amine was only mildly active. The results recorded in Table I are in general agreement with those of Wawzonek, Ponseti, Shepard, and Wiedenmann (3) and further extend the list of related structures which are non-lathyrogenic.

TABLE I.—LATHYROGENIC ACTIVITY OF CERTAIN COMPOUNDS

Compound	Concn., %	No. of Rats	Days Fed	Activity[a]
A. Fed in Dog Chow				
β-aminopropionitrile	0.35	2	18	4+
	1.0	1	13	Lethal
Aminoacetonitrile sulfate	0.04	2	23	0
	0.07	2	23	1+
	0.15	2	26	4+
	0.30	2	7	Lethal
	0.53	2	4	Lethal
γ-Aminobutyronitrile HCl	0.35	1	15	0
	0.70	1	30	0
bis-(β-cyanoethyl)amine	0.25	4	28	2±
β-hydroxypropionitrile	0.5	2	23	0
	1.0	2	23	0
Cyanoacetic acid	1.0	3	28	0
Cyanoacetamide	1.0	2	27	0
Propylamine HCl	2.0	3	23	0
Ethylenediamine 2 HCl	1.0	2	28	0
B. Fed in Nontoxic Pea Ration[b]				
Aminoacetonitrile sulfate	0.03	1	22	P
		1	31	2+
	0.06	2	18	3+
	0.11	2	15	4+
β-aminopropionitrile	0.025	6	36	1+
	0.05	1	17	P
	0.05	5	30	2+
	0.10	2	25	3+

[a] 1+–4+ indicates range of skeletal deformity from mild to very severe. P indicates paralysis without marked skeletal change.

[b] Methanol extracted Singletary peas 70%, Vitamin A, D, E fortified corn oil 5%, B-complex fortified glucose 3%, Hubbel, Mendel, and Wakeman salts 2%, glucose to make 100%.

It had previously been observed that methanol extraction at atmospheric pressure yielded a more toxic extract than did extraction *in vacuo*. It had also been noted that toxic fractions were prone, under conditions favorable to mild hydrolysis, or alcoholysis, to develop a substance which gave a beautiful blue color with ninhydrin. When the amide structure of the naturally occurring compound became evident and it was found that β-aminopropionitrile, itself lathyrogenic, yielded such a blue color with ninhydrin, it was evident that a simple qualitative test for the free and amide-combined forms of the β-aminopropionitrile could be developed. The details of such a test are given in the experimental section.

This procedure was applied to the seed of various legumes. Positive results for the presence of β-aminopropionitrile in combined form were obtained with seed of *L. odoratus* and *L. pusillus*. No preformed free β-aminopropionitrile could be detected in either case. Neither free β-aminopropionitrile nor γ-glutamyl-β-aminopropionitrile could be detected in seed of *L. latifolius*, *Vicia sativa*, *Canavalia ensiformis*, *Phaseolus coccineus*, *Trifolium incarnatum* or *Medicago sativa*.

We have previously commented (1) that the toxic substance of the Singletary pea did not produce symptoms as described for certain other members of the genus (4). The evidence presented above for *L. latifolius* lends further support to our statement that at least two different toxic compounds will eventually be found in various members of the genus.

We were intrigued by the fact that others (2, 3) using our isolation procedure obtained an apparently pure toxic substance from *L. odoratus*, while we obtained from the Singletary pea a toxic product contaminated with apparently related compounds. During the development of the qualitative test for the presence of free or the γ-glutamyl combined forms of β-aminopropionitrile, it became evident that the contaminants were not in the methanol extracts, whether prepared at atmospheric pressure or *in vacuo*, of the *L. odoratus* seed. They were easily demonstrable, however, in methanol extracts of the Singletary pea seed prepared *in vacuo*. Their occurrence seems to be a species difference. It is suggested that these related contaminants may be the corresponding amide and acid since they yield the same amino acids

on hydrolysis. It is of interest in this regard that both indolylacetic acid and indolylacetonitrile have been found in plant extracts under conditions which indicate both are present in the same tissue (5).

EXPERIMENTAL

Final Purification.—The crystalline toxic material obtained as previously described (1) was examined by ascending paper chromatography using Whatman No. 1 paper, butanol:acetic acid:water (5:1:4) as mobile phase and 0.15% ninhydrin in acetone as the color reagent. Three zones were found. These were separately eluted with water and each was hydrolyzed by boiling with 6 *N* HCl. Each of the three hydrolysates was chromatographed as before. All yielded spots which behaved as did known samples of glutamic acid and β-alanine.

Two grams of the crystalline material was dissolved in the minimum amount of water and placed on a large column ($2^1/_4$ in. x $20^1/_2$ in.) of standard grade Whatman powdered cellulose which had been equilibrated with butanol saturated with water. Such a butanol solution was used as developer. Six liters of the developing solution was passed through the column at a rate of 100 ml. per hour. Eluate fractions of 100 ml. each were collected, and each was examined by paper chromatography as before. Fractions shown by such examination to contain the same zone, uncontaminated by the other materials present in the original mixture, were then pooled.

The three pooled solutions were concentrated *in vacuo*. Dry matter was determined for each solution. Each concentrated solution was then evaporated over an individual portion of ground Dog Chow at a level equivalent to 1.5% of the original crystalline material. Only the Dog Chow carrying material from the slowest travelling zone gave evidence of being toxic when fed to weanling albino rats. Accordingly, this was the only zone crystallized. This was done by further evaporating *in vacuo* until crystallization began, chilling and filtering. Recovery averaged 60% of the solid introduced into the column. M. p. 192–192.8°. This crystallized, chromatographically homogeneous product is hereinafter referred to as the toxic material.

Anal.—Calcd. for $C_5H_{11}N_3O_3$: C, 48.24; H, 6.53; N, 21.1. Found: C, 48.18; H, 6.73; N, 20.78.

An infrared absorption spectrum, obtained through the courtesy of Dr. J. L. E. Erickson, showed a strong band at 4.45 μ which was best ascribed to the presence of a nitrile group.

Calculated alkali-labile nitrogen for one nitrile group per mole: 7.03%, one-third of the total nitrogen. Found: 6.95%, one-third of the total nitrogen.

Calculated carboxyl carbon for one—$CHNH_2CO$-OH group per mole: C, 6.03. Found by Van Slyke manometric ninhydrin (6) method: C, 6.01.

Identification of Constituents.—The 2,4-dinitrophenyl derivative of the toxic material was prepared by Sanger's method (7). This was hydrolyzed with 6 *N* HCl and the ether extract of the hydrolysate was examined by paper chromatography. Whatman No. 1 paper previously impregnated with phthalate buffer of *p*H 6 and air-dried was used. Tertiary amyl alcohol saturated with the buffer solution was used as mobile phase. Only one spot was visible either in daylight, yellow color, or under ultraviolet illumination. This spot displayed the characteristics of a known 2,4-dinitrophenyl derivative of glutamic acid.

The residue left after extraction of the hydrolysate with ether was examined by paper chromatography. The presence of β-alanine was indicated. A 3,5-dinitrobenzoyl derivative was prepared which melted at 200–201°. The same melting point was found for a mixture with the 3,5-dinitrobenzoyl derivative of an authentic sample of β-alanine.

Five hundred milligrams of the toxic material was hydrolyzed for four hours at 100° with 4 ml. of 6 *N* HCl. The resulting solid was dissolved in the minimum quantity of water and HCl was introduced until precipitation occurred. The precipitate was removed, dried and found to melt at 189–190°. A mixture with authentic glutamic acid hydrochloride gave the same melting point.

Test for Amide-bound β-Aminopropionitrile:—Five grams of comminuted seed was placed in a paper extraction thimble in a Soxhlet extractor and extracted with petroleum ether (b. p. 35–37°) for twelve hours. The thimble and contents were air-dried, then extracted with absolute methanol (100 ml. initial volume) for twelve hours at atmospheric pressure in a Soxhlet extractor. The γ-glutamyl-β-aminopropionitrile was alcoholyzed under such conditions.

The unconcentrated methanol extract was examined by paper chromatography. Whatman No. 1 paper was used with butanol:acetic acid:water (5:1:4) as mobile phase. The color reagent was 0.15% ninhydrin in acetone.

The presence of β-aminopropionitrile in the extract was indicated by the development of a clear marine-blue spot with R_f of 0.30 at 25°. A confirmatory test consisted of allowing the chromatogram to stand for forty-eight hours, bleaching by exposure to HCl vapor, standing for one hour in the air and examining under ultraviolet light. The spot previously marine blue now exhibited an intense golden fluorescence.

Aminoacetonitrile, which gave a yellow spot with R_f 0.25 under these conditions, was not found in any of the seeds examined.

Test for Free β-Aminopropionitrile.—A similar extraction procedure with absolute methanol *in vacuo* at 25–30° yielded an extract which did not contain free β-aminopropionitrile unless the compound was initially present in the free state in the material extracted. The γ-glutamyl-β-aminopropionitrile was not alcoholyzed at this lower extraction temperature.

CONCLUSIONS

1. The toxic entity of the Singletary pea is γ-glutamyl-β-aminopropionitrile. It occurs with two apparently related, nontoxic compounds.

2. Aminoacetonitrile, β-aminopropionitrile and bis-(β-cyanoethyl) amine produced the typical symptoms of lathyrism when fed to albino

rats. The toxicities decreased in the order named.

3. A qualitative test for the presence in seed of amide-bound or free β-aminopropionitrile, was developed.

4. This test indicates that neither free nor γ-glutamyl bound β-aminopropionitrile is present in seed of *Lathyrus latifolius*. Our previous statement that at least two toxic entities will be found in the genus is reaffirmed.

REFERENCES

(1) Dupuy, H. P., and Lee, J. G., THIS JOURNAL, **43**, 61 (1954).
(2) Schilling, E. D., and Strong, F. M., *J. Am. Chem. Soc.*, **76**, 2848(1954).
(3) Wawzonek, S., Ponseti, I. V., Shepard, R. S., and Wiedenmann, L. G., *Science*, **121**, 63(1955).
(4) Lewis, H. B., Fajans, R. S., Esterer, M. B., Shen, Chao-Wen, and Oliphant, M., *J. Nutrition*, **36**, 537(1948).
(5) Henbest, H. B., Jones, E. R. H., and Smith, G. F., *J. Chem. Soc.*, 3796(1953).
(6) Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.* **141**, 627(1941).
(7) Sanger, F., *Biochem. J.*, **39**, 507(1945).

Ultrasonic Extraction of *Cinchona succirubra**

By W. F. HEAD, Jr., H. M. BEAL,† and W. M. LAUTER‡

Twenty kilocycle and 450 kc. ultrasonic extraction and ultrasonic maceration, followed by Soxhlet extraction, has been compared to and shown to be more efficient than Soxhlet extraction alone. Possible structural changes of the alkaloids by ultrasonic waves were also studied in each of the solvents employed at both frequencies. Chromatography of the alkaloidal extracts obtained from each solvent was performed to determine selectivity or completeness of extraction.

ONLY A SMALL PERCENTAGE of ultrasonic investigations have been directed toward aiding drug extraction. The manifestations of ultrasonics, some of which are cavitation, agitation, and penetration, would seem to favor extraction considerably. The present investigation was initiated on this basis.

Gomez and Matas found that fresh *Strophanthus gratus* seeds lost about 50 per cent of their strophanthin content upon storage (1). Application of ultrasonics, however, did not improve the yield of strophanthin and actually diminished it in alcoholic solutions.

Haul, Rust, and Lutzow studied the effect of ultrasonic energy on partition extraction (2). A light gasoline phase was bubbled through a methanol phase containing dissolved phenanthrene. With a gasoline flow rate of about 2 ml. per second and 1 to 9 watts ultrasonic energy, the improvement in extraction amounted to 76 per cent.

Schultz and Klotz extracted cinchona with sonic and ultrasonic waves, and reported that sonic waves aid the extraction while ultrasonic waves have no beneficial action (3). It was noted, however, that if the drug had not been softened, ultrasonics was advantageous. Their equipment was a 5-cm. diameter plate transducer delivering 20 watts at 2.4 mc., and the solvent employed was dilute formic acid. This frequency is rather high to be used in drug extraction since most extraction work has been done in the range of 25 to 600 kc. Cavitation is also at a minimum at these higher frequencies.

To obtain a more complete extraction picture, cinchona was extracted under alkaline, acidic, and neutral conditions, using chloroform with ammonia, 5 per cent hydrochloric acid, and tetrahydrofuran-water azeotrope (TWA) respectively. Ultrasonic extraction was effected at 20 kc. and 450 kc. and comparisons were made with Soxhlet extractions under the same conditions.

EXPERIMENTAL

Standardization of the Crude Drug.—A single batch of *Cinchona succirubra*, in a number 60 powder, was used throughout this investigation. The percentage of total alkaloids was determined on several samples by the method of the N. F. IX (4). The average alkaloidal yield was 5.73 per cent.

Ultrasonic Equipment.—A Rich-Roth ultrasonic generator, model 400, and a Rich-Roth Sonicell®, type L 250 were used in this work. The generator

* Received September 9, 1955, from the College of Pharmacy, University of Florida, Gainesville.
Abstracted in part from a thesis presented to the graduate council of the University of Florida by W. F. Head, Jr. in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
Presented to Section Np, A.A.A.S., Atlanta meeting December, 1955.
† Assistant Professor of Pharmacy, University of Florida.
‡ Professor of Pharmaceutical Chemistry, University of Florida.

is a variable frequency unit and produces approximately 400 watts of electrical energy over a range from 10 to 1200 kc. per second.

The Rich-Roth Sonicell® is a metal lined and jacketed, hermetically sealed, water cooled, multi-frequency ultrasonic transducer. The crystal is a piezoelectric hollow cylinder made of barium titanate and is cut to match the output frequencies of the generator. It is mounted upon, and acoustically coupled to a copper treatment chamber. There is approximately 280 cm.^{2} of ultrasonic generating area. In order to resonate with different generator frequencies, potentials are applied to the length, thickness, or circumference of the crystal. For this work, the length potential producing approximately 20 kc., and the thickness potential producing approximately 450 kc. were used.

The complete unit was then calibrated calorimetrically for power output. It was found that at 20 kc. the output was 26.1 watts, while at 450 kc. the energy was 81.4 watts in the copper treatment chamber.

Quantitative Extraction Studies.—Soxhlet extractions were made with chloroform and 5 per cent HCl for one, three, five, and seven hours. Soxhlet extractions with TWA have been performed previously (5). It was decided to extract with ultrasonics for one-half, one, one and one-half, and three hours. All extractions were done in duplicate and ultrasonic extractions were carried out in appropriate size polyethylene bottles.

Samples of cinchona, weighing 15 Gm., were allowed to macerate with a solution composed of 20 ml. ether, 10 ml. alcohol, and 5 ml. ammonium hydroxide for fifteen minutes. Extractions were then effected with chloroform. In addition, ultrasonic maceration with the same ammonia-ether-alcohol solution, at 20 kc., for fifteen minutes, followed by Soxhlet extraction was performed.

The extraction and maceration scheme for 5 per cent HCl was similar to that of chloroform with the exception that one hour maceration periods with this acid were used. Ultrasonic maceration with HCl was effected at 450 kc. It should be pointed out that all Soxhlet extractions required the addition of an antifoam agent to prevent excess foaming. In this investigation, "DC Antifoam A"[1] was used.

Extractions with TWA were performed on 5-Gm. samples of cinchona in the absence of alkalinity or acidity and without a maceration period. The TWA was prepared by distilling commerical tetrahydrofuran with approximately 10 per cent water. High boiling mineral oil was also added to prevent the concentration of peroxides from becoming too large in the still residue. Since ultrasonic waves have been known to cause oxidation (6, 7), ascorbic acid, 0.25 per cent, was added to the TWA to prevent peroxide formation during extractions.

The TWA and chloroform extracts were assayed, following evaporation of the solvent, by the official assay procedure for the residue as if it were crude cinchona (4). The HCl extracts were made basic with ammonia and extracted thoroughly with chloroform. This chloroform was then evaporated and the residue treated in the same manner as above.

The results of these extractions are shown in Fig. 1, 2, and 3.

[1] Dow Corning Corporation, Midland, Mich.

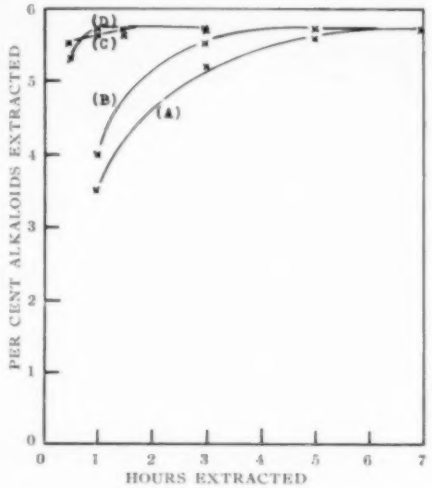


Fig. 1.—Chloroform extraction using various procedures. (A) Soxhlet extraction. (B) 20 kc. ultrasonic maceration followed by Soxhlet extraction. (C) 20 kc. ultrasonic extraction. (D) 450 kc. ultrasonic extraction.

Effect of Ultrasonic Waves on Structure of Alkaloids.—Ultrasonic waves in several instances have been known to effect structural changes in otherwise stable compounds. The best examples of this are the depolymerizations of polystyrene and hydroxyethylcellulose. The oxidation effects have been previously mentioned. Consideration of the oxidizable portions of all four major cinchona alkaloids indicated the need of determining if any changes in structure were effected during ultrasonic extraction.

Optical Rotation and Infrared Studies.—A series of 1 per cent solutions of each alkaloid in each solvent used in extractions were prepared. Cinchonine, in chloroform, however, was only 0.5 per cent due to its solubility. Since ascorbic acid is optically active and is decomposed by ultrasonic waves, it was omitted from the TWA during this phase of investigation.

These solutions were then measured for optical rotation using a Carl Zeiss polarimeter with a 1-dcm. tube. Specific rotations were then calculated.

Fifteen milliliters of each alkaloid in each solvent was added to polyethylene tubes and irradiated at both 20 kc. and 450 kc. ultrasonic frequency for three hours. At the end of this time the solutions were quantitatively transferred to small graduates and solvent was added to make the original volume of 15 ml. Specific rotations were obtained as before.

There was no change in specific rotations from controls to irradiated samples in any solvent at either frequency.

Ultrasonically irradiated samples of alkaloids were obtained in the following order: quinine from 20 kc. treated 5 per cent HCl, quinidine from 20 kc. irradiated TWA, cinchonine from 450 kc. treated chloroform, and cinchonidine from 450 kc. irradiated TWA solutions.

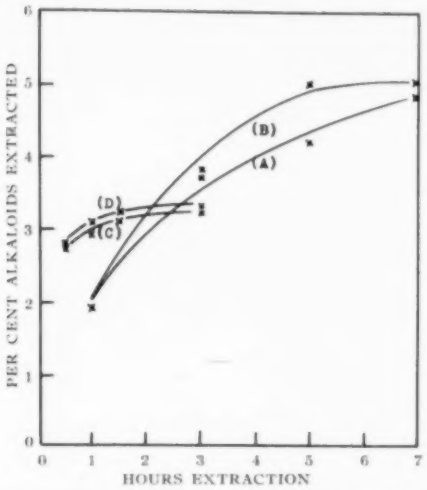


Fig. 2.—Five per cent hydrochloric acid extraction using various procedures. (A) Soxhlet extraction. (B) 450 kc. ultrasonic maceration followed by Soxhlet extraction. (C) 20 kc. ultrasonic extraction. (D) 450 kc. ultrasonic extraction.

The infrared spectrum for each alkaloid which had been irradiated was obtained and was identical with the corresponding non-irradiated samples.

Chromatography of Alkaloidal Extracts.—Lussman, Kirch, and Webster have described a method of paper partition chromatography for the four major cinchona alkaloids (8). This procedure was used in examining the extracts isolated in this study. All extracts were obtained from the assay procedure final residue. Since these alkaloids, at this point, are relatively pure, the possibility of other extracted plant material hindering their adsorption is held to a minimum. All extracts were from 30 minute, 20 20 kc. ultrasonic extractions. In addition, controls containing all four major cinchona alkaloids were developed.

Quinidine, quinine, and cinchonidine were identified, by means of their R_f values, from the chloroform extracts. The R_f values obtained were 0.56, 0.50, and 0.33, in order. No other bands appeared on this chromatogram

From the 5 per cent HCl extract, four bands were developed. Three of these bands corresponded to quinidine, quinine, and cinchonidine, with R_f values of 0.57, 0.48, and 0.34, respectively. The other band did not correspond to cinchonine and was probably due to one of the minor cinchona alkaloids. Its R_f value was 0.13, whereas cinchonine has an R_f of 0.42 (8).

It has been previously shown that TWA is selective for quinine and cinchonine when extracted in a Soxhlet apparatus (5). Using the same chromatographic procedure and an ultrasonic extract, it was found that a total of 5 bands existed. Quinidine, quinine, and cinchonine were easily identified by R_f values of 0.60, 0.48, and 0.43 respectively. The fourth band corresponded to cinchonidine (R_f=0.32), however, this band was also slightly fluorescent. The color produced by the developing reagent was also different from that of cinchonidine controls. The fifth band had a low R_f value of 0.14 and was probably the same alkaloid which appeared in the HCl extracts.

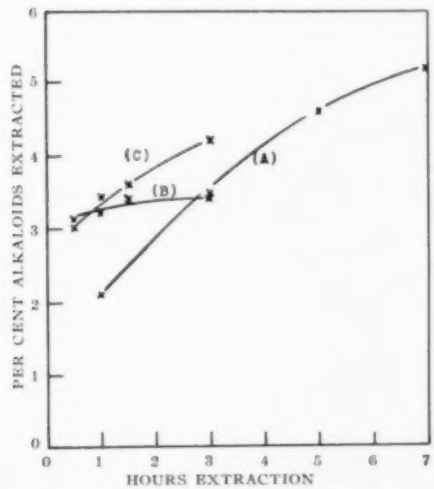


Fig. 3.—Tetrahydrofuran-water azeotrope extraction using various procedures. (A) Soxhlet extraction. (B) 20 kc. ultrasonic extraction. (C) 450 kc. ultrasonic extraction.

CONCLUSIONS

1. It was noted that the ultrasonic extraction curves for chloroform and 5 per cent HCl were very similar in configuration. Ultrasonic chloroform curves, however, were considerably higher than ultrasonic HCl curves and ultrasonic TWA curves were intermediary. The Soxhlet curves using each of these solvents were significantly lower than the respective ultrasonic extraction curves. Using a Soxhlet apparatus, the best solvents in order of effectiveness were chloroform, TWA, and 5 per cent HCl.

2. From the results obtained in optical rotation studies and infrared spectra examinations, it is evident that ultrasonic waves of 20 kc. and 450 kc. frequencies, do not affect the cinchona alkaloids structurally.

3. Chromatography of the irradiated cinchona extracts from chloroform and HCl revealed the presence of quinidine, quinine and cinchonidine. From TWA extracts, quinine, quinidine, cinchonine, and possibly cinchonidine were demonstrated.

REFERENCES

(1) Gomez, C. G., and Matas, L. C., *Med. Colonial, (Madrid)*, **13**, 401(1950).

(2) Haul, R., Rust, H. H., and Lutzow, J., *Naturwissenschaften*, **37**, 523(1950).
(3) Schultz, O. E., and Klotz, J., *Arzneimittel-Forsch.*, **4**, 325(1954).
(4) "National Formulary," 9th ed., Mack Publishing Co., Easton, Pa., 1950, p. 146.
(5) Campo, J. M., "Extraction of *Cinchona succirubra* Pavon et Klotzsch with Tetrahydrofuran and its Mixtures," Ph. D. Dissertation, University of Florida Library, 1955, pp. 12, 33.
(6) Schmitt, F. O., Johnson, C. H., and Olson, A. R., *J. Am. Chem. Soc.*, **51**, 370(1928).
(7) Liu, S., and Wu, H., *ibid.*, **56**, 1005(1934).
(8) Lussman, D. J., Kirch, E. R., and Webster, G. L., THIS JOURNAL, **40**, 368(1951).

Extraction of *Cinchona succirubra* with Tetrahydrofuran and its Mixtures*

By J. M. CAMPO and L. G. GRAMLING

A study has been made on the extraction of *Cinchona succirubra* with tetrahydrofuran, tetrahydrofuran-water azeotrope (5.3% water) (1), tetrahydrofuran-chloroform azeotrope, and tetrahydrofuran-water solution (15% water v/v) for the purpose of establishing alkaloidal selectivity of the solvents under examination. A tetrahydrofuran-benzene azeotrope was prepared and its composition was determined by index of refraction measurements. This azeotrope was also tested for its ability to extract the cinchona alkaloids. The highest degree of alkaloidal extraction was observed with the tetrahydrofuran-water azeotrope using Soxhlet extraction. A method has been described whereby cinchonine alkaloid can be isolated in reasonably pure form from a tetrahydrofuran-water azeotrope extract of the crude drug. The selectivity of the tetrahydrofuran-water azeotrope for quinine and cinchonine alkaloids is reported and discussed.

IN AN INVESTIGATION of various furans as solvents for the extraction of belladonna root, Carkuff (2) reported selectivity for *l*-hyoscyamine by Soxhlet extraction of the powdered drug with tetrahydrofuran-water azeotrope. Other investigations have shown that tetrahydrofuran in combination with water shows a greater selectivity for caffeine than either solvent alone (3). In a previous investigation the authors reported results on the extraction of ergot with tetrahydrofuran and its mixtures and observed the highest degree of alkaloidal extraction with tetrahydrofuran-chloroform azeotrope (4). These findings suggested a further investigation of tetrahydrofuran mixtures in the extraction of medicinal constituents of crude drugs.

A batch of red cinchona bark powder (#60) was used for this investigation. Because commercial tetrahydrofuran contains a stabilizer for the purpose of preventing the formation of peroxide, it was distilled just prior to extraction. All original extractions in this investigation were performed in the absence of alkalinity. Thus the ability of the solvents to extract the alkaloids as they occur naturally could be determined.

* Received September 9, 1955, from College of Pharmacy, University of Florida, Gainesville.
Abstracted from a dissertation presented to the Graduate Council of the University of Florida by Joseph M. Campo in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
The authors wish to thank E. I. du Pont de Nemours & Company, Wilmington, Delaware, for their generous supply of tetrahydrofuran required for this investigation.

EXPERIMENTAL

Preliminary Investigations

Surface Tension of Tetrahydrofuran-Water Solutions.—It was decided to make a study of the effect produced on the surface tension of tetrahydrofuran by the addition of increasing increments of water. Low surface tension would tend to favor more efficient extraction of the alkaloids (5). A series of v/v solutions of water in tetrahydrofuran was prepared and the surface tension of each solution determined with a du Nouy Tensiometer. The surface tension curve is shown in Fig. 1. The curve shows that concentrations of water in tetrahydrofuran up to approximately 50% do not appreciably increase the surface tension of the solvent.

Solubility of Cinchona Alkaloids in Tetrahydrofuran-Water Solutions.—Samples of the principal cinchona alkaloidal bases; quinine, quinidine, cinchonine, and cinchonidine, each weighing 200 mg., were dissolved in a series of tetrahydrofuran-water solutions, measuring from a buret the minimum quantity of solvent required to effect complete solution of the alkaloids. The solubility curves thus obtained are shown in Fig. 2. The curves show that maximum solubility for each of the alkaloids is achieved at approximately 15% water content. Although the alkaloids occur in the crude drug as salts of organic acids, solubility studies on the free bases were conducted so as to give some indication of selectivity.

Preparation and Determination of Composition of Tetrahydrofuran-benzene Azeotrope.—A mixture of 75 ml. freshly distilled tetrahydrofuran and 75 ml. benzene was distilled using a steam bath. A constant b. p. fraction was collected at 73.5 – 74°. Because neither tetrahydrofuran nor benzene boils

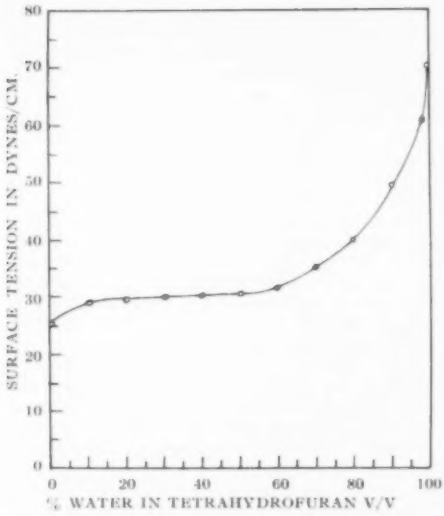


Fig. 1.—Surface tension of tetrahydrofuran-water solutions.

at this temperature, azeotrope formation is indicated. The composition of this azeotrope was determined in the same manner as previously reported for the tetrahydrofuran-chloroform azeotrope (4). The refractive index curve (Fig. 3) shows that the azeotrope contains 35.2% benzene in tetrahydrofuran.

Quantitative Extraction Studies

Standardization and Procedure.—The total alkaloidal content of the crude drug used throughout this investigation was determined by the N. F. IX method (6). A series of such determinations revealed that the total alkaloidal content of the crude drug was 5.67%.

Because tetrahydrofuran is miscible with water (7), this solvent cannot be applied to partition extraction with acid solutions as in the case of ether and chloroform which are immiscible with water. It was decided, therefore, that the extent of alkaloidal extraction by a given procedure could be best determined by official assay of the marcs after extraction with the solvent under examination. The degree of alkaloidal extraction could then be determined by difference, based on the total alkaloidal content of the crude drug prior to extraction.

Mechanical Agitation.—Accurately weighed samples of crude cinchona (5 Gm.) were agitated in a mechanical shaker for two hours with 100 ml. pure tetrahydrofuran. This was the same shaker used in the official determinations. The machine produces 200 oscillations per minute with a one-inch stroke. At the end of the two-hour extraction period the marc was filtered off, washed thoroughly with fresh solvent, and dried. The marc was then assayed by the N. F. IX method. An average of 0.1544 Gm. residual alkaloid was found per 4 Gm. of the original crude drug. The degree of alkaloidal extraction was 31.5% (see Table I).

Soxhlet Extraction.—Accurately weighed samples of crude cinchona (5 Gm.) were extracted for varying periods of time by means of Soxhlet extractors, adjusting the rate of extraction to one cycle every five minutes. The solvents employed were tetrahydrofuran, tetrahydrofuran-water azeotrope, tetrahydrofuran-chloroform azeotrope, and tetrahydrofuran-benzene azeotrope. In each extraction 100 ml. of the solvent under examination was used. The marcs were then thoroughly dried and assayed by the N. F. IX method (6). The results are recorded in Table I.

Tetrahydrofuran-Water Solution (15% v/v).—It has been shown by the solubility curves of the cinchona alkaloids in tetrahydrofuran-water solutions that a maximum solubility is reached at approximately 15% water content (see Fig. 2). Surface tension at this concentration is shown to be at a minimum (see Fig. 1). It was therefore decided to devise an extraction apparatus by which solutions of this concentration could be efficiently employed. Soxhlet extraction could not be applied since the solvent coming in contact with the drug would have the water content of the azeotrope (5.3% water).

The apparatus is shown in Fig. 4. It consisted of a small percolator (*a*) with a solvent delivery tube (*b*) running from the lower orifice of the percolator upward and into the top of a separatory funnel (*c*) placed alongside the percolator. With the stopcock of the separatory funnel closed, vacuum applied in the separator by means of a vacuum line (*d*) would cause percolation of the extract into the separator. A vacuum control valve (*e*) was placed in the vacuum line so that the rate of percolation could be controlled and varied. An electrically controlled mechanical stirrer (*f*) was placed in the percolator. The shaft of the stirrer ran through a lubricated rubber seal (*g*) at the top of the percolator. This arrangement was used to prevent evap-

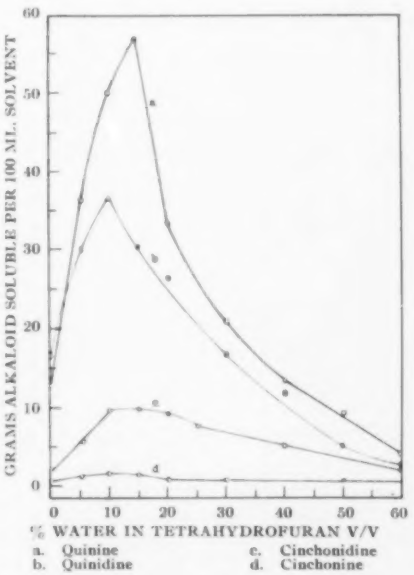


Fig. 2.—Solubility of cinchona alkaloids in tetrahydrofuran-water solutions.

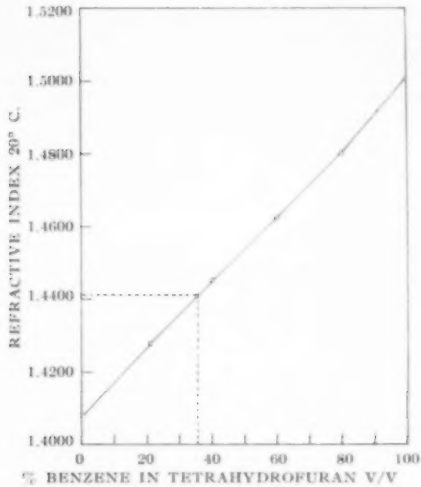


Fig. 3.—Refractive index of tetrahydrofuran-benzene solutions.

oration of the highly volatile solvent. The stirring rod shaft was connected to the motor shaft with a rubber tube universal joint (*h*) to compensate for motor vibrations. With this arrangement the drug could be vigorously agitated with the solvent prior to percolation. In the official assay procedure mechanical shaking is employed (6).

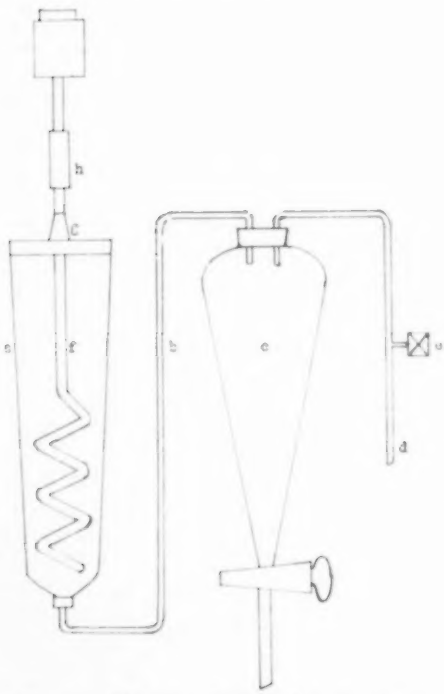


Fig. 4.—Diagram of extraction apparatus.

Samples of crude cinchona (5 Gm.) were placed in the percolator and 100 ml. of tetrahydrofuran containing 15% water was added. The system was closed and mechanical agitation was started. Agitation was continued for three hours. The motor was then stopped and the top of the percolator was opened to admit air for vacuum percolation which was made to proceed dropwise by means of the vacuum control valve. After the marc was exhausted of solvent, the sides of the percolator and stirrer were washed with 50 ml. of fresh solvent and agitation resumed for a half hour to wash the marc. The washings were vacuum percolated until the drug was exhausted of solvent. This washing procedure was repeated. Assay of the marcs for residual alkaloid revealed that the average degree of alkaloidal extraction was 80.6%.

Table I represents a summary of the data obtained by the extraction of the crude drug by the various solvent combinations employed. Although the tetrahydrofuran-water azeotrope gave the highest results, it is interesting to note that the selectivity of the tetrahydrofuran-chloroform azeotrope for the cinchona alkaloids is very low. Very good results in the extraction of ergot alkaloids were obtained with the tetrahydrofuran-chloroform azeotrope (4). This difference is noteworthy in regard to selective extraction.

TABLE I.—SUMMARY OF RESULTS OBTAINED FROM EXTRACTION OF CRUDE CINCHONA BY VARIOUS SOLVENTS

Solvent	Procedure	Extraction Time, hrs.	Extraction, %
THF[a]	Agitation	2	31.5
THF	Soxhlet	4	27.8
THF	Soxhlet	6	31.5
THF-Water Azeotrope	Soxhlet	4	64.8
THF-Water Azeotrope	Soxhlet	6	86.6
THF-Water 15% v/v	Percolation	....[b]	80.6
THF-$CHCl_3$ Azeotrope	Soxhlet	6	6.5
THF-Benzene Azeotrope	Soxhlet	6	23.9

[a] Tetrahydrofuran
[b] See text.

Isolation and Identification of Cinchonine

Isolation.—A tetrahydrofuran-water azeotrope extract representing a comparatively larger quantity of crude drug was prepared so that isolation studies could be facilitated. A battery of four Soxhlet extractors was assembled. A specially constructed elongated steam bath with four openings at appropriate intervals was used as a source of heat for these extractors. Samples weighing 15 Gm. each were placed in the extractors and moistened with 25 ml. of tetrahydrofuran-water azeotrope. Each sample was extracted for four hours. At the end of the extraction period all four thimbles were replaced with thimbles containing fresh drug and each sample was macerated with fresh solvent as before. Eight extractions were carried out for each of the four extractors making a total of thirty-two extractions. The extracts when combined rep-

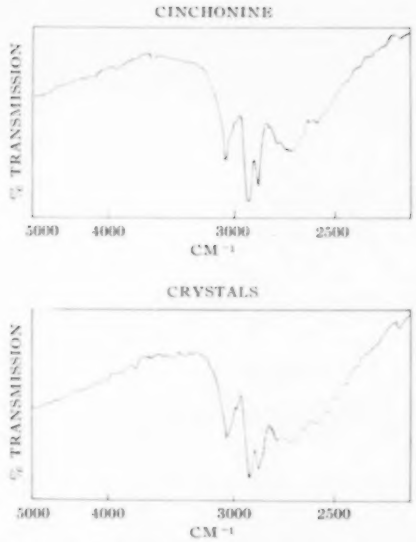


Fig. 5.—Comparison of infrared absorption spectra for known cinchonine and crystals.

resented 480 Gm. of crude drug or slightly less than half a kilogram.

The combined extracts were transferred to a large evaporating dish and the solvent evaporated on a steam bath. The dark brown residue remaining was dried in an oven at 100°. The dried mass was then ground into a powder using a mortar. The ground powder was further dried in the oven at 100°.

A sample of the powdered residue weighing 40 Gm. was extracted with three 20-ml. portions of hot water. The combined aqueous solutions were cooled and filtered. The filtrate was rendered distinctly alkaline to litmus with stronger ammonia test solution and extracted with five 20-ml. portions of benzene. Slight crystallization was observed in the benzene fractions. The combined benzene extracts were then extracted with five 20-ml. portions of 5% sulfuric acid solution. The combined acid solutions were made distinctly alkaline to litmus with stronger ammonia test solution and extracted with five 20-ml. portions of benzene. The combined benzene extracts were then concentrated on a steam bath. During this concentration procedure it was observed that crysals were forming in the hot solvent. At this point heating was stopped and the solution allowed to cool and stand overnight to complete crystallization. The crystals were then filtered off, dried, and further purified by recrystallization from benzene.

Examination of Crystals.—The purified crystals obtained by the above extraction procedure were examined under the microscope using low power. Very definite uniform long needles were observed. The uniform appearance of these crystals indicated that the compound was in a reasonably pure state. A few crystals dissolved readily in 5% sulfuric acid solution. A portion of this solution produced a precipitate immediately upon the addition of one drop of Valser's reagent. The crystals were found to be insoluble in water and alkaline solutions. They dissolved readily in the common organic solvents. The data thus far correspond to the properties of alkaloids.

Paper Partition Chromatography of Crystals.—In order to determine which of the cinchona alkaloids was present in this fraction, it was decided to make chromatograms from the crystals and compare them with chromatograms of the four principal cinchona alkaloids. The procedure employed for this determination is that described by Lussman *et al.* (8). The paper cylinder used in the present determination was 23 cm. high and 7 cm. in diameter. A very small quantity of the crystals was dissolved in 95% ethanol and the resulting solution was spotted along a straight line running one inch from and parallel to the bottom of the paper cylinder. When the ethanol was completely evaporated from the paper, the cylinder was placed in the developing chamber containing the cylohexanol and diluted hydrochloric acid developing solutions. After thirty-six hours of development the cylinder was removed from the chamber and thoroughly air dried. When the paper was examined under ultraviolet light no fluorescent bands were detected. A control chromatogram of the four principal cinchona alkaloids which was developed simultaneously with the unknown showed pronounced ultraviolet bands for both quinine and quinidine. The unknown chromatogram was then cut into strips $^3/_4$ inch wide. These strips were then sprayed with diluted potassium iodoplatinate solution (8). A pronounced dark blue band developed in the lower half of the chromatogram. The advance of this band measured 9.2 cm. from the point of origin. On the basis of the advance of the column front which measured 20 cm. from the point of origin, it was calculated that the R_f value for this unknown band was 0.46. The corresponding band in the control chromatogram was cinchonine, the R_f value of which was calculated to be 0.45. The literature reports the R_f value for cinchonine as 0.42 ± 0.05 (8) under the identical conditions of this determination.

Infrared Absorption Curve.—As a further check in establishing the identity of the cinchonine alkaloid it was decided to make an infrared absorption study of the alkaloid. Solutions were prepared of the alkaloid under examination and of a known sample of cinchonine. The infrared absorption curves obtained are compared in Fig. 5. This comparison further helps to establish the identity of cinchonine alkaloid.

Melting Point of Cinchonine Alkaloid.—A few crystals of the alkaloid were dried in an oven overnight at 100°. During melting point determination sublimation was observed at 220°. The compound melted over the range 255–260°. These values for sublimation and melting point correspond to the values reported in the literature for cinchonine alkaloid (9).

Alkaloidal Selectivity of Tetrahydrofuran-Water Azeotrope.—The tetrahydrofuran-water azeotrope extract was examined chromatographically employing the same procedure previously described (8). Only quinine and cinchonine alkaloids were detected in the chromatogram.

Various attempts were made to isolate cin-

chonidine as the insoluble tartrate and quinidine as the insoluble hydriodide from the tetrahydrofuran-water azeotrope extract, but these attempts were not successful. The absence of cinchonidine and quinidine alkaloids in the extract further helped to establish alkaloidal selectivity of the solvent for quinine and cinchonine alkaloids.

REFERENCES

(1) Gramling, L. G., private communication (1954).
(2) Carkhuff, E. D., and Gramling, L. G., THIS JOURNAL, **41**, 660(1952).
(3) "Tetrahydrofuran," E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.
(4) Campo, J. M., and Gramling, L. G., THIS JOURNAL, **42**, 747(1953).
(5) Brochmann-Hanssen, E., *ibid.*, **43**, 27(1954).
(6) "The National Formulary," 9th ed., Mack Publishing Co., Easton, Pa., 1950, p. 146.
(7) "New Products Bulletin No. 4," E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.
(8) Lussman, D. J., Kirch, E. R., and Webster, G. L., THIS JOURNAL, **40**, 368(1951).
(9) Heilbron, M., "Dictionary of Organic Compounds," Vol. I, Oxford University Press, New York (1934) p. 342.

The "Melting Point" of Suppositories.

Device for Testing the Resistance of Fatty-Base Suppositories to Deformation*

By S. REZNEK

Fatty-base suppositories, when ideally formulated, melt at body temperature and yet retain their firmness and shape in ordinary storage. It is desirable to have a test procedure which will show the melting behavior of a formulation as a finished suppository. Since a static melting test gives a misleading evaluation of the behavior of a suppository, a procedure is described wherein, by pneumatically applying a slight pressure to a suppository held at a specified temperature, its behavior under use conditions may be better evaluated.

IT IS PRESUMED that the medicating action of fatty-base suppositories becomes effective only after the suppository has melted to a more or less liquid consistency. The melting range should therefore not be above body temperature. On the other hand, it should not be so low as to allow melting under ordinary shelf storage conditions.

Cocoa butter (Theobroma Oil) is probably the most common single constituent of the fatty base. In regard to its melting characteristics, the U. S. P. XV states only that Theobroma Oil "is usually brittle at temperatures below 25°." Since this temperature is often exceeded in ordinary storage, higher melting fats or waxes are incorporated to raise the melting range to levels at which the suppository is more likely to retain its shape. However, because of the variability in cocoa butter, as well as the variable effect of the other ingredients on the melting range, it is difficult to predict the melting behavior of the finished suppository. In addition, as is well known, the "melting point" of cocoa butter, as of other fats, slowly increases after initial solidification from the melted state and testing of new formulations should take this behavior into account.

* Received September 24, 1955, from Sterling-Winthrop Research Institute, Rensselaer, New York.

EXPERIMENTAL

In the process of examining a series of formulations in which the cocoa butter-wax ratio was varied through a considerable range, there was found to be little or no relation between the melting behavior at body temperature (taken as 37°) and the composition of the base. The difficulty lay largely in determining when the suppository had effectively melted. A suppository frequently showed, by its glistening surface appearance, together with the fact that it readily deformed under slight finger pressure, that it had reached a melted state. If left undisturbed, however, it retained its original shape even after days at 37°.

From this behavior, it appeared that the criterion of melting behavior for a suppository should be its tendency to deform under slight pressure at a given temperature rather than during static storage at that temperature. The device described below was developed to measure this tendency.

Recognizing that a static melting test is not significant, Malangeau (1) describes an arrangement for determining the mechanical resistance of cocoa butter. In Malangeau's procedure, a cylinder of cocoa butter of stated dimensions is molded and cooled. A 500-Gm. weight is then applied to the

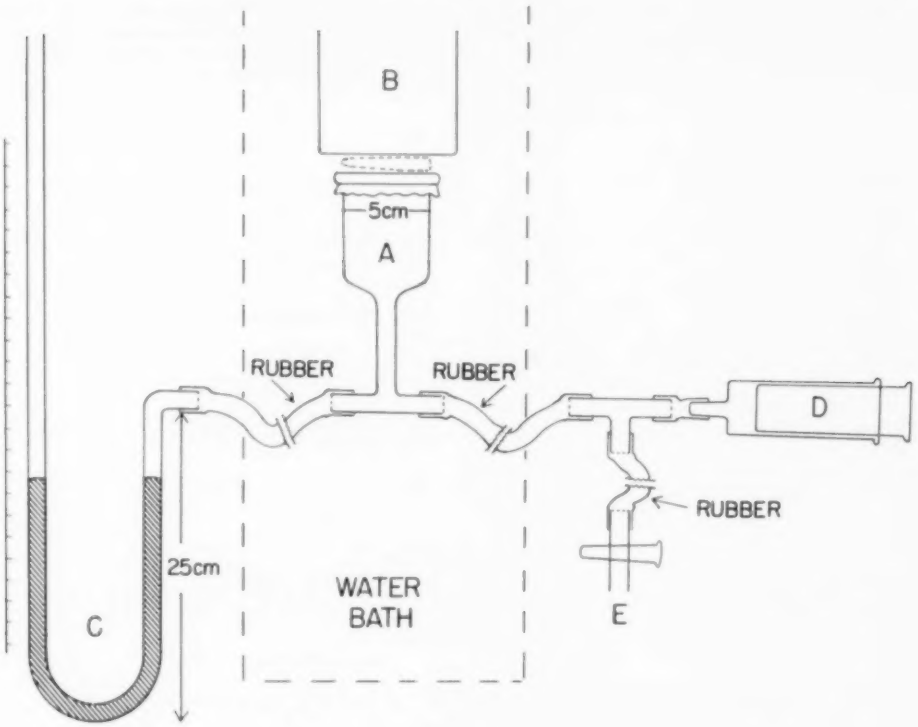


Fig. 1

long axis of the cylinder and the temperature gradually raised until the cylinder collapses. Alternatively, the cylinder is held at a fixed temperature (30°) and the weight gradually increased.

Rather than use a cylinder of arbitrary dimensions specially cast for the test, it is more to the point to test the "mechanical resistance" of actual suppositories. Moreover, considering the low order of pressure required to deform a fatty mass such as a suppository, the pressure is more conveniently controlled by pneumatic means than by Malangeau's technique of using weights or mercury.

As shown in the schematic drawing (Fig. 1), the apparatus is designed to apply and measure the pressure required to deform a suppository which has been brought to the desired temperature in a water bath. The suppository, encased in a cellophane envelope closed on all sides except for a small opening to allow escape of air as the temperature is raised, is laid on the thin rubber dam cemented airtight to the periphery of the glass cup *A*. *B* is a glass cylinder, with closed flat bottom and open top, filled with water to the level of the bath when the whole apparatus is lowered into the bath. *B* is adjusted to barely touch the suppository as indicated by the development of approximately 1 cm. pressure in the water manometer *C*, with stopcock *E* closed. *A* and *B*, which are attached to a common support, are then lowered into the bath and *E* opened to release pressure developed in *A* by the heat of the bath. When the system has equilibrated to bath temperature (20 minutes has been found sufficient) *E* is closed and the plunger of *D* (30-cc. Luer syringe) previously set at about $^3/_4$ open position, is slowly depressed until the level of the open-end manometer is raised 10 cm. from its point of zero pressure (equivalent to 20 cm. H_2O pressure, or 20 Gm./cm.^{2} applied on the rubber dam). This pressure has been found to distinguish between relatively soft and relatively hard masses at 37°, in that the former are completely flattened between the rubber dam and *B*, while the latter retain more or less of their original shape.

Table I gives the results of tests upon seven experimental and three commercial lots of suppositories, four suppositories having been tested from each lot. There was appreciable variation between individual samples in the lots; the variation may have been real, arising either from non-uniform mixing or separation of ingredients on cooling, or may have been a consequence of irregular application of pressure during the test on the torpedo-shaped suppositories (Samples *A*–*G*, and 2). Nevertheless, the average ratings show a regular progression in softness as the proportion of hardening agent (wax) is decreased; also, as expected from the proportions used (2), the effect of spermaceti is to decrease the hardness.

The applied pressure and the rating scale (as given in Table I) are both arbitrary; experience with different formulations, sizes, and shapes of suppositories may suggest variations in the test conditions. Applied pressure, bath temperature, and time of immersion may all be adjusted according

TABLE I.—BEHAVIOR OF COCOA BUTTER BASE SUPPOSITORIES AT 37° ON APPLICATION OF 20 GM./CM.² PRESSURE

Identification (Formulas)	Composition (Ratio of base components) Cocoa Butter	White Wax	Sperma-ceti	Appearance after applying 20 Gm./cm.² at 37° Ratings of Individual Suppositories[a]
Exp. A	5	1	....	1, 2, 3, 3
Exp. B	6	1	....	2, 2, 2, 2
Exp. C	7	1	....	3, 3, 4, 4
Exp. D	8	1	....	3, 4, 4, 4
Exp. E	9	1	....	2, 5, 5, 5
Exp. F	5	1	0.33	4, 4, 4, 4
Exp. G	5	..	0.33	6, 6, 6, 6
Comm. 1	Not known			4, 5, 5, 5
Comm. 2	Not known			4, 4, 4, 4
Comm. 3	Not known			4, 4, 4, 4

[a] Legend: 1. Little or no deformation; 2. Compressed to ³/₄ original thickness; 3. Compressed to ¹/₂ original thickness; 4. Compressed to ¹/₄ original thickness; 5. Completely deformed; 6. Liquefied.

to the judgment of the test laboratory. After the most suitable test procedure has been determined by the development laboratory, the same conditions would be applicable to routine control.

SUMMARY

1. A simple apparatus is described for determining the resistance of suppositories to deformation at a specified (e. g., body) temperature.

2. A rating scale is suggested to describe the behavior of the suppositories under pressure at a specified temperature.

REFERENCES

(1) Malangeau, P., *Ann. pharm. franç.*, **6**, 50(1948).
(2) Scoville, "Art of Compounding," 8th ed., Blakiston, Philadelphia, Pa., 1951, p. 348.

Digitalis Studies by the U.S.P. XIV Method of Assay I.*

Seasonal Variation in Response of the Pigeon to Digitalis

By R. A. SACHS, J. D. HIGHSTRETE, and M. L. PABST

Assay data on the U. S. P. XIV digitalis reference standard, obtained over a four-year period, have been examined statistically to show that a seasonal variation exists in the response of pigeons to the reference standard. Investigation was made of the effect of seasonal variation on relative potency by the comparison of data obtained at thirty-day and sixty-day intervals. It was found that the seasonal variation had little effect if the U. S. P. XIV assay completion limit of thirty days was observed. Greater variation occurred when the time interval was increased to sixty days.

REPORTED STUDIES (1, 2) show a marked seasonal variation in the response of frogs to digitalis and ouabain in that the animals were more resistant to these drugs during the months of June, July, August, September, and October. This marked seasonal variation was not found when the cat was used as the test animal (2, 3).

Our assay results on the U. S. P. XIV digitalis reference standard, obtained over a period of four years, were examined to determine if a seasonal variation existed in the response of pigeons to digitalis. All tests on the reference standard met the U. S. P. XIV requirements. For purposes of comparison the lethal doses (LD) were computed as cc. of tincture digitalis per Kg. of test animal.

RESULTS

The average LD for each test on the reference standard is shown in Table I. In general the average LD was greater during the summer months, indicating that the animals were more resistant to digitalis at this time. The change in LD values was not always distinct nor did it always occur at the same time each year. However, the peak of resistance always occurred during the months of August or September.

The average LDs in Table I were subjected to statistical tests to determine if the seasonal differences were significant. In order to do this the LD values were divided into two groups. Group I included the results obtained during the months of May, June, July, August, September, and October. Group II included the results obtained during the remaining six months of the year.

* Received July 5, 1955, from the Biological Control Laboratories of The Upjohn Company, Kalamazoo, Mich.
The authors are indebted to Dr. O. S. Carpenter for advice regarding statistical procedures.

TABLE I.—AVERAGE LETHAL DOSES OF U. S. P. XIV DIGITALIS REFERENCE STANDARD
(cc. Tincture Digitalis per Kg.)

Group I	Group II	1950	1951	1952	1953
	Jan.	...	0.861	0.871	0.833
	Feb.	...	0.815	0.852	0.796
	Mar.	0.750	...	0.889	0.833
	Apr.	0.794	0.787	0.861	0.785
May	...	...	0.898	...	0.910
June	...	...	0.861	0.815	...
July	...	...	0.907	0.871	0.880
Aug.	...	0.872	0.954	0.917	0.935
Sept.	...	0.906	0.944	0.926	0.907
Oct.	...	...	...	0.871	...
	Nov.	0.754	0.815	0.833	0.861
	Dec.	...	...	0.815	0.944

The mean LD, $\bar{x}_1$, for Group I ($N_1 = 16$) was 0.898 cc. tincture per Kg., and the mean LD, $\bar{x}_2$, for Group II ($N_2 = 19$) was 0.829 cc. tincture per Kg. The mean LD for all results, Group I and Group II ($N = 35$) was 0.861 cc. tincture per Kg. The difference, d, of the means ($\bar{x}_1 - \bar{x}_2$) was 0.069 cc. tincture per Kg.

An analysis of variance was used to determine whether the variances of the two groups were significantly different and whether the two means $\bar{x}_1$ and $\bar{x}_2$ were significantly different.

TABLE II.—ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total	34		
Between groups	1	0.0419	0.0419
Within groups	33	0.0596	0.0018
Group I	15	0.0189	0.0013
Group II	18	0.0407	0.0023

F (Group I to Group II) = 0.0023/0.0013 = 1.77.

F (between groups to within groups) = 0.0419/0.0018 = 23.28.

The F value (Group I to Group II) was not significant at the 5% level, therefore the variances of the two groups were not significantly different. The F value (between groups to within groups) was greater than the F table value at 1%, therefore the difference in LD means $\bar{x}_1$ and $\bar{x}_2$ was highly significant. This shows that a seasonal variation in the response of pigeons to digitalis occurred with the data presented in Table I.

The t-test is probably used more frequently than the F-test to determine differences between two means. For this reason the t-test was also used to test for differences between the two average LD means $\bar{x}_1$ and $\bar{x}_2$. The result of the t-test should agree with the result of the F-test as the variance ratio F is equal to t^2.

$$t = d/s_d = 0.069/0.014 = 4.9$$

The value of t was greater than the table value at 1% which indicates that the two means differ significantly. This is in agreement with the result of the F-test.

The U. S. P. XIV assay procedure for digitalis requires that the assay be completed within a period of thirty days. By interpretation, thirty days may be allowed to elapse between tests on the standard and the test preparation. Because a seasonal variation was apparent from the data in Table I, we wished to determine what effect the change in LD values would have on relative potencies. The average LDs of standard were compared to obtain potency estimates. Comparisons were made in the following manner: (*a*) The interval between average LD determinations was 30 days ± 4 days. For each potency estimate both average LDs were in the same group, either Group I or Group II (within groups). (*b*) Same as (*a*) except one of the average LDs was in a different group (between groups). (*c*) Same as (*a*) except the time interval was 60 days ± 5 days. (Two exceptions were potency estimates at 49 and 50 days.) (*d*) Same as (*b*) except the time interval was 60 days ± 5 days.

The standard error, S. E., was calculated for each average LD in Table I. The average assay S. E. was found to be 0.0428 or 4.28%. The potency estimates obtained by the comparison of average LDs of standard are plotted in Fig. 1 with the limits of ±2 S. E. (8.56%) indicated.

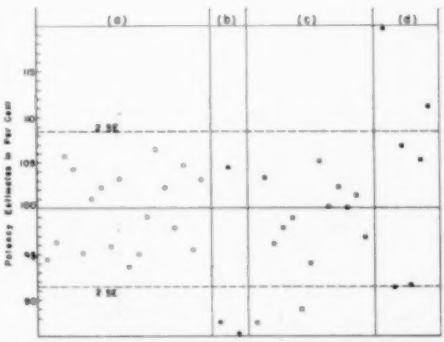


Fig. 1.—Potency estimates for thirty and sixty day intervals.

It was possible to calculate 21 potencies when the time interval was 30 days ± 4 days and 2 or 9.5% fell outside of ± 2 S. E. limits. These were divided in Fig. 1 into (*a*) within groups and (*b*) between groups. There were 18 potency estimates in (*a*) and all fell within the ±2 S. E. limits. Only 3 potency estimates could be determined for (*b*) and 2 or 67% fell outside of the ± 2 S. E. limits. This indicated that valid potency estimates were obtained when the time interval did not exceed 30 days as specified in the U. S. P. digitalis assay procedure. Although there were only 3 potency estimates in (*b*), between groups, it suggested that more variation may occur when there is a marked change in LD values due to seasonal variation.

Nineteen potency estimates were calculated when the time interval was 60 days ± 5 days and 5 or 26% exceeded the ± 2 S. E. limits. There were 13 potencies in (*c*), within groups, and 2 or 15% exceeded the ± 2 S. E. limits. There were 6 potencies in (*d*) and 3 or 50% exceeded the ± 2 S. E. limits. Therefore, when the time interval was increased from 30 to 60 days more of the estimated

potencies exceeded the ± 2 S. E. limits. This indicated that a time interval as long as 60 days for completion of tests is not desirable for the assay of digitalis.

The data and results in this report are based only on tests using the U. S. P. XIV digitalis reference standard. For this information to be applicable to the U. S. P. digitalis assay procedure, we must assume that test preparations of digitalis and the reference standard respond in a similar manner. This assumption was made with the adoption of the reference standard.

SUMMARY

1. Seasonal variation in the response of pigeons to the U. S. P. digitalis reference standard exists in the data presented.

2. Potency estimates were within acceptable limits of experimental error in nearly all cases when the 30-day U. S. P. XIV restriction was observed. In a few cases variation exceeding acceptable limits was introduced at the time of seasonal shifts in average lethal doses.

3. Potency estimates exceeding acceptable limits of experimental error occurred more frequently when the time interval for the completion of tests was increased to 60 days.

REFERENCES

(1) Swoap, O. F., and Pabst, M. L., THIS JOURNAL, **29**, 59(1940).
(2) Chen, K. K., Henderson, F. G., and Robbins, E. B., *J. Pharmacol. Exptl. Therap.*, **107**, 131(1953).
(3) Bliss, C. I., and Hanson, J. C., THIS JOURNAL, **28**, 521(1939).

Digitalis Studies by the U. S. P. XIV Method of Assay II.*

Stability of Crude Digitalis Leaf

By R. A. SACHS, J. D. HIGHSTRETE, and M. L. PABST

The stability of crude digitalis leaf was studied for two years, using the U. S. P. XIV pigeon method of assay. The data showed: (*a*) a loss of potency in the first twelve months, (*b*) no difference in stability between the ground or whole leaf forms, and (*c*) possibly greater stability when storage was in sealed rather than unsealed containers.

NUMEROUS STUDIES (1–10), on the stability of digitalis leaf report that the leaf is stable for periods up to several years when tested by the frog and the cat methods of assay. Other studies (11–14), report that the leaf is not stable for long periods when tested by the same assay methods. Some batches of leaf may be more stable than others and this may account for some of this disagreement. Also, lack of precision in the assay methods employed may account for the apparent deterioration in some cases.

Since these earlier publications, the assay procedure has been markedly improved. A U. S. P. digitalis reference standard has been adopted, limits have been set for the number of injections, the rate of injection has been standardized, and statistical procedures have been employed for potency estimation and validity tests. These improvements have been incorporated in the U. S. P. XIV assay procedure. By use of the U. S. P. XIV method, we wished to study the stability of a crude digitalis leaf under conditions of storage applicable to the storage of relatively large quantities of leaf to be used for manufacturing purposes.

EXPERIMENTAL

A representative sample (30 pounds) of a commercial lot of dried digitalis leaf was divided into two equal parts. One part was ground with a small ball mill and screened through a thirty-mesh sieve; the other part was kept as the whole leaf. Individual samples for each assay of the ground leaf and the whole leaf were stored under identical conditions in open paper sacks, unsealed cardboard containers, and sealed metal containers at room temperature (22–23° and relative humidity 20–55%). Similar samples were stored in sealed metal containers in the refrigerator (4°).

The initial potency of the leaf was found to be 17.4 U. S. P. XIV digitalis units per gram of leaf, this figure was based on the average of three complete assays (16.7, 16.9, and 18.6 digitalis u./Gm.). The supplier reported a potency of 17.6 U. S. P. XIV digitalis units per gram of leaf. Moisture determinations were made at the time of the original assay and at the time of each succeeding assay; these varied from 5.4 to 9.1%. All assays met the U. S. P. XIV requirements.

* Received July 5, 1955, from the Biological Control Laboratories of The Upjohn Company, Kalamazoo, Mich.
The authors are indebted to Dr. O. S. Carpenter for advice regarding statistical procedures.

RESULTS

The potency values for the samples of leaf stored for 12, 18, and 24 months under the test conditions are shown in Table I.

TABLE I.—POTENCY EXPRESSED AS U. S. P. XIV DIGITALIS UNITS PER GM. LEAF

Leaf Form	Storage Container	12 Mo.	18 Mo.	24 Mo.	Average
Whole	Paper sack	12.0	14.3	13.6	13.1
Whole	Cardboard	11.7	14.9	12.2	12.9
Whole	Metal, room temp.	13.0	15.5	15.2	14.6
Whole	Metal, refrig.	14.0	14.6	14.5	14.4
Ground	Paper sack	13.4	13.3	14.1	13.6
Ground	Cardboard	14.5	13.2	14.0	13.9
Ground	Metal, room temp.	15.2	14.9	13.5	14.5
Ground	Metal, refrig.	14.1	16.3	15.2	15.2
	Average	13.5	14.6	14.0	14.0

The average potency including both the whole leaf and the ground leaf under the various conditions of storage for 12, 18, and 24 months was 14.0 digitalis units per Gm. of leaf. The difference, d, between the original potency of 17.4 and 14.0 was 3.4 units.

The t-test was used to determine if these two values were significantly different ($t = d/S_d = 3.40/0.676 = 5.03$). The value of t was greater than the table value of t at 1%, which indicated that the two potency estimates differed significantly. This is evidence that there was a loss of potency following the original assay.

The potencies in Table I were tested by an analysis of variance to determine whether there was a loss of potency related to leaf form, storage container, and time of storage.

TABLE II.—ANALYSIS OF VARIANCE OF TABLE I

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
L: Whole or ground leaf	1	1.60	1.60
T: Time of storage	2	5.18	2.59
S: Storage container	3	9.29	3.10
S_1: Paper vs. cardboard	1	0.003	0.003
S_2: Metal R.T. vs. metal ref.	1	0.160	0.160
S_3: Sealed vs. unsealed	1	9.137	9.137
LS: Interaction	3	0.98	0.33
ST: Interaction	6	1.02	0.17
LT: Interaction	2	4.21	2.10
Residual error	6	7.54	1.26
Total	23	29.82	

The residual error variance was used in estimating the significance of leaf form (L), storage container (S), and time of storage (T).

($F_L = 1.60/1.26 = 1.27$; $F_T = 2.59/1.26 = 2.06$; $F_S = 3.10/1.26 = 2.46$; $F_{S_1} = 0.003/1.26 = 0.002$; $F_{S_2} = 0.160/1.26 = 0.127$; $F_{S_3} = 9.137/1.26 = 7.25$)

The F test for the leaf form, F_L, was not significant at the 5% level which shows no real difference in the stability between the whole leaf and the ground leaf.

The F test for the time of storage, F_T, was not significant at the 5% level which shows no real loss of potency during the 12 to 24 months storage period. As the t-test showed a real loss of potency following the original assay, it is evident that the loss occurred during the first year of storage.

In the analysis of variance for the method of storage, F_S, the types of storage were divided into three groups: F_{S_1}, paper sack versus cardboard container, F_{S_2}, metal container at room temperature versus metal container at refrigerator temperature, and F_{S_3}, sealed container versus unsealed container. The F test for F_{S_1} was not significant at the 5% level which indicates that there is no real difference in stability of the leaf when stored in paper sacks or cardboard containers. The F test for F_{S_2} was not significant at the 5% level which shows that the leaf is equally stable in metal containers at room temperature and at refrigerator temperature, the F test for F_{S_3} is significant at the 5% level but not significant at the 1% level. This suggests that the leaf may be more stable when stored in sealed metal containers than when stored in unsealed paper sacks or cardboard containers. Because the F test for F_{S_3} was not significant at the 1% level, further work would be necessary to substantiate such a conclusion.

SUMMARY

1. The batch of digitalis leaf studied showed a loss in potency during the first twelve months of storage. No further loss in potency occurred during the twelve to twenty-four month storage period.

2. No difference in stability between the ground leaf or whole leaf form of digitalis was observed during the twenty-four month test period.

3. The data suggest that digitalis leaf may be more stable when stored in sealed rather than in unsealed containers.

REFERENCES

(1) Hale, W., *Bull.* No. 74, *Hyg. Lab.* U. S. Marine Hospital Service, Washington (1911); *Chem. Abstr.*, **5**, 1824 (1911).

(2) Sharp, J. G., and Lancaster, J., *Pharm. J.*, **86**, 102 (1911), *Chem. Abstr.*, **5**, 1973(1911).

(3) Hatcher, R. A., and Eggleston, C., *Am. J. Pharm.*, **85**, 203(1913).

(4) Haskell, C., and Miller, F. A., THIS JOURNAL, **3**, 306(1914).

(5) Gold, H., and DeGraff, A. C., *J. Am. Med. Assoc.*, **90**, 1016(1928).

(6) Haag, H. B., and Hatcher, R. A., *ibid.*, **93**, 26(1929).

(7) Haag, H. B., and Hatcher, R. A., *Am. J. Pharm.*, **101**, 474(1929).

(8) Rowe, L. W., and Pfiefle, H. W., THIS JOURNAL, **25**, 855(1936).

(9) Edmunds, C. W., Moyer, C. A., and Shaw, J. R., *ibid.*, **26**, 290(1937).

(10) Haag, H. B., *Am. J. Pharm.*, **110**, 456(1938).

(11) Schmidt, J. M., and Heyl, F. W., THIS JOURNAL, **91**, 425(1919).

(12) Wastensen, H., *Svensk. Farm. Tid.*, **27**, 433(1923); *Chem. Abstr.*, **17**, 3747(1923).

(13) Gronberg, J., *Pharm. Zentralhalle*, **64**, 403(1923); C. C. B., **95**, 1413(1924).

(14) Christensen, B. V., and Smith, R. B., THIS JOURNAL, **27**, 841(1938).

Sabadilla Alkaloids V.*

Vanilloylcevine

By DAVID M. STUART† and LLOYD M. PARKS

A new crystalline alkaloid, vanilloylcevine, $C_{38}H_{49}NO_{11}$ has been isolated from Sabadilla seed, *Schoenocaulon officinale*, and characterized as the vanillic acid ester of cevine. Vanilloylcevine exhibits moderate hypotensive activity in dogs.

PREVIOUS STUDIES on sabadilla alkaloids in this laboratory have used hot extraction procedures to obtain the alkaloids from the seed. In these studies there were obtained cevadine, veratridine (veratroylcevine) and the new alkaloid, sabatine (1–4). Because of the heat lability of cevine and the ease of hydrolysis of ester alkaloids containing this alkamine (5–8), a cold extraction was used in the present study.

By means of the extraction procedure described in the Experimental Part the following fractions were obtained: Fraction, Sa(Skelly B-extract); Sb(benzene extract); Sc(chloroform extract); Sd, Se, and Sf(alcohol extract). The ultraviolet absorption spectra of these fractions showed that Sa, Sb and Sc contained considerable amounts of veratridine-like alkaloids while the other fractions showed no characteristic spectra.

Fraction Sc was further separated into a benzene-soluble and a benzene-insoluble fraction. The benzene-soluble fraction was then subjected to a modified seven-plate single-withdrawal countercurrent distribution, using chloroform as the mobile phase and 0.5 *M* citrate buffer at *p*H 4.25 as the stationary phase, which resulted in the distribution pattern shown in Fig. 1.

The ultraviolet absorption spectrum of the material in buffer plate 0 showed a maximum at 238 mμ, indicating the presence of sabadilline (3). Spectra of buffer plates 1 and 2 showed maxima at 292 and 262 and minima at 282 and 238 mμ, indicating the presence of veratridine. Buffer plates 3 through 7 showed no characteristic absorption. All of the chloroform plates, 0 through 7, showed the characteristic absorption for veratridine.

The above information indicated that Fraction Sc, benzene-soluble, had been separated roughly into four components. Since partition chromatography (2) showed veratridine to be a major component of chloroform plates 0 through 7 and cevadine a major component of buffer plates 3 through 7, it was possible to shorten the separation procedure by using a modified two-plate single-withdrawal countercurrent distribution. When this was done, using chloroform as the mobile phase and 0.5 *M* citrate buffer at *p*H 4.25 as the stationary phase, Fraction Sc, benzene soluble, was separated into three parts, namely, (A) material in the buffer plate 0; (B) buffer plates 1 and 2; and (C) material in the chloroform plates 0 through 7.

Partition chromatography of the material in buffer plates 1 and 2, using 0.5 *M* citrate buffer at *p*H 4.25 as internal phase and chloroform as eluant, resulted in the elution of only a small amount of material which was shown to be cevadine. When the eluant was changed to 25 per cent ethanol in chloroform alkaloidal material having a veratridine-like absorption was eluted but it was difficult to separate from some of the internal phase which was eluted along with it.

With 0.5 *M* citrate buffer at *p*H 5.0 as internal phase and chloroform as eluant, partition chromatography of the material in buffer plates 1 and 2 gave the results shown in Fig. 2. The material eluted in fractions 20 to 40 crystallized from acetone as blunt needles and was recrystallized from absolute ethanol as white silky needles which were identified as vanilloylcevine, m. p. 257.5–258.5° (decompn.).

The ultraviolet absorption spectra of vanilloylcevine and veratridine are shown in Fig. 3 and their infrared spectra in Fig. 4.

Vanilloylcevine was also found in (A), buffer plate 0 of the modified countercurrent distribution at *p*H 4.25, during attempts to separate the sabadilline contained therein. Vanilloylcevine was separated from the other material in (A) by means of a modified two-plate countercurrent distribution, using chloroform and 0.5

* Received July 2, 1955, from the School of Pharmacy, University of Wisconsin, Madison.

This investigation was supported in part by a research grant, H-1083, from the National Heart Institute of the National Institutes of Health, U.S. Public Health Service, and in part by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

† Fellow, American Foundation for Pharmaceutical Education, 1954–1955. Present address: University of Texas, College of Pharmacy, Austin, Texas.

This paper is adapted from the prize-winning manuscript submitted by David M. Stuart in the Central Region, 1955 Lunsford Richardson Pharmacy Awards competition.

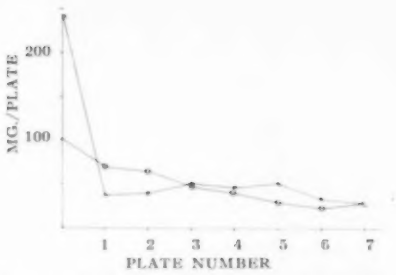


Fig. 1.—Modified countercurrent distribution of Fraction Sc (benzene soluble). × = buffer plates; O = chloroform plates.

M phosphate buffer at *p*H 6.75. By partition chromatography of the material remaining in the chloroform plates, using 0.5 *M* phosphate buffer at *p*H 6.50 as internal phase and chloroform as eluant, vanilloylcevine was eluted in the first fifteen fractions, as shown in Fig. 5. These conditions for obtaining vanilloylcevine are applicable in the absence of veratridine and cevadine. In their presence, however, a partition column buffered at *p*H 5.0 is necessary.

Since vanilloylcevine was only partially hydrolyzed by the mild alkaline procedure of Kupchan (8) it was necessary to separate the unhydrolyzed ester from the alkamine by a nine-plate countercurrent procedure, using chloroform as the mobile phase and 0.5 *M* phosphate buffer at *p*H 6.75 as the stationary phase. This resulted in the distribution shown in Fig. 6. The alkamine was recovered from plates 0 through 2 and the unhydrolyzed ester from plates 3 through 9. Vanilloylcevine was also found to be incompletely hydrolyzed after four hours refluxing with sodium ethoxide.

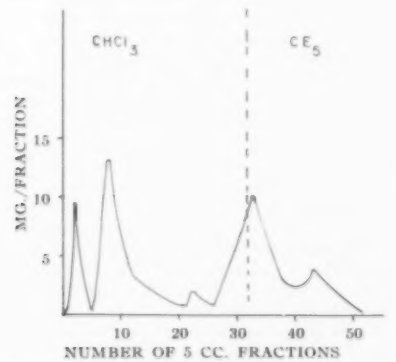


Fig. 2.—Partition chromatography of (B) with 0.5 *M* citrate buffer at *p*H 5.0 as internal phase. CE_5 = chloroform-ethanol (95:5).

The acid portion of the ester was identified as vanillic acid by analysis and mixed melting point test. The alkamine form identified was cevagenine, by comparison of its infrared spectrum with that of a sample of cevagenine obtained by an identical hydrolysis procedure from cevine. Both spectra were identical with that reported by Stoll (5). Recent reports have shown (8–10) that this alkamine may be obtained in one of three forms, namely veracevine, cevagenine

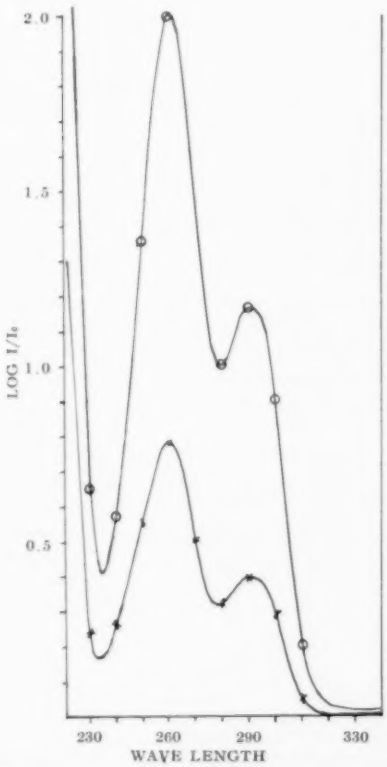


Fig. 3.—Ultraviolet absorption spectra of vanilloylcevine (x); c = 0.104 Gm./L.) and veratridine (O; C = 0.043 Gm./L).

or cevine, depending upon the severity of the hydrolysis conditions. In view of the fact that cevagenine was obtained under the conditions used by us, this new alkaloid appears to be the vanillic acid ester of cevine. Methylation of the vanilloylcevine with diazomethane converted it to veratridine.

Marion, Ramsay, and Jones (11) reported the isolation, by saponification of crude veratrine, of a minor alkaloid, m. p. 266–270.5°, whose infrared spectrum (12) showed the presence of a carbonyl group. A sample of their alkaloid,

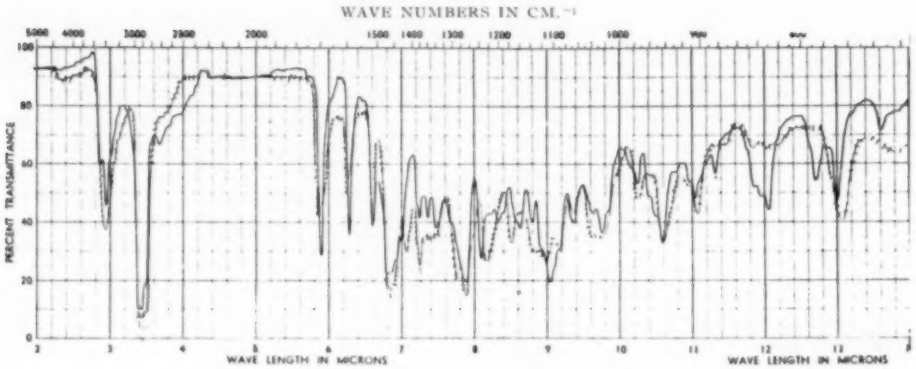


Fig. 4.—Infrared spectra of vanilloylcevine (——) and veratridine (++++) in Nujol.

kindly furnished us by Dr. Leo Marion, was found to be identical to our vanilloylcevine by mixed melting point (257.5–8.5°), ultraviolet and infrared absorption spectra. Although Marion, *et al.* did not state the conditions under which they saponified crude veratrine it is apparent that our observation on the difficulty of hydrolysis of vanilloylcevine explains their finding this alkaloid among the saponification products.

When injected into the femoral vein of nembutal-anesthetized dogs, vanilloylcevine, in doses of 0.1 mg./Kg. caused an immediate 50 per cent reduction in blood pressure which gradually returned to normal in 70 minutes. In doses of 0.2 mg./Kg. it caused an initial sudden increase in blood pressure followed, in 15 minutes, by a decrease to less than 50 per cent of normal, and gradually returning to normal at the end of two hours.

EXPERIMENTAL

Extraction.—Ground sabadilla seed (15.4 Kg.) was moistened with Skelly B, which had been saturated with concentrated ammonia, packed in a percolator, and allowed to macerate for twenty hours. Percolation with Skelly B, at the rate of approximately 3 cc. per minute, was continued for forty-five days and the 45 liters of percolate then reduced *in vacuo* to about 8 L. The yellow concentrate, in 2-L. portions, was shaken with portions of 10% acetic acid until the washings were negative to Mayer's reagent (total washings, 6 L.). The acid extract was made basic with concentrated ammonia to produce a white precipitate which, when filtered and dried, amounted to 270 Gm. (fraction Sa). Washing the basic filtrate with chloroform to a negative Mayer's test and evaporation of the chloroform *in vacuo* yielded 17 Gm. of dark brown residue [fraction Sa (1)].

Percolation of the air dried marc was continued with benzene, saturated with ammonia, until the percolate gave a negative Mayer's test (50 L.). The benzene percolate, when treated in the same manner as the Skelly B percolate, yielded 28 Gm. of precipitate (fraction Sb). From the alkaline filtrate there was obtained an additional 3 Gm. [fraction Sb (1)].

Percolation of the air dried marc was attempted with chloroform. When the percolate refused to flow at a constant rate a maceration procedure was resorted to, allowing the drug to soak for forty-eight hours before each draining. In this way 141 L. of macerate was collected before a negative Mayer's test was given. The chloroform macerate was concentrated to 1.5 liters *in vacuo* and the concentrate poured, with stirring, into 2 L. of 6% acetic acid. After the two layers were separated the chloroform layer was shaken with 250-cc. portions of the acid until the washings gave a negative Mayer's test (total washings 21 L.). The acid extract, in 1-L. portions, was cooled to 0–10°, 300 cc. of concentrated ammonium sulfate solution (70 Gm./100 cc.) added, and the *p*H of the solution raised to 9–10 by addition of concentrated ammonia. It was then washed with four successive 500-cc. portions of chloroform for the removal of alkaloids. The total chloroform extract (12 L.) was dried over anhydrous sodium sulfate and distilled *in vacuo* to yield a residue of 234 Gm. (fraction Sc).

Fraction Sc was stirred with 2.5 L. of benzene for one and one-quarter hours, the suspension filtered and the residue washed with 1.5 L. of benzene. Distillation *in vacuo* of the combined filtrate and washings yielded 187 Gm. of light yellow solid (fraction Sc, benzene-soluble). The benzene-insoluble portion, upon drying, amounted to 47 Gm. (fraction Sc, benzene-insoluble).

Percolation of the air dried marc was continued with alcohol until the percolate gave a negative Mayer's test (100 L.). The alcohol percolate was concentrated to 1 liter and poured, with stirring, into 8 L. of water. The dark brown precipitate when filtered, washed with water and dried, yielded 95 Gm. of dark brown material (fraction Sd). The filtrate was made alkaline with concentrated ammonia and the resulting yellow precipitate, when filtered and dried, amounted to 11 Gm. (fraction Se). Washing the basic filtrate with chloroform to a negative Mayer's test and evaporation of the chloroform *in vacuo* yielded an additional 30 Gm. (fraction Sf).

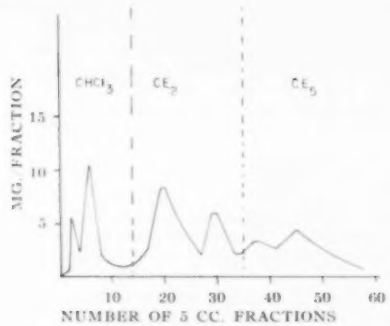


Fig. 5.—Partition chromatography of (A) with 0.5 *M* phosphate buffer at *p*H 6.5 as internal phase.

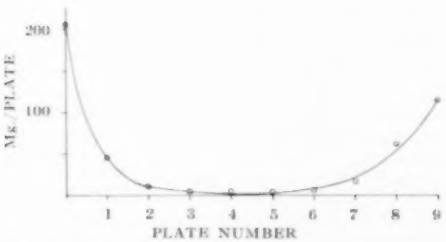


Fig. 6.—Countercurrent distribution of vanilloylcevine hydrolysis mixture.

Modified Countercurrent Distribution of Fraction Sc, Benzene Soluble.—A one-Gm. sample was dissolved in 50 cc. of chloroform and shaken with 50 cc. of 0.5 *M* citrate buffer, *p*H 4.25, for two minutes. The chloroform layer was then shaken with seven additional 50-cc. portions of buffer. A fresh 50-cc. portion of chloroform was then shaken with each of the eight buffer layers in turn and this procedure was repeated with six additional 50-cc. portions of chloroform, resulting in eight buffer plates and eight chloroform plates. Each chloroform plate was dried over anhydrous sodium sulfate, evaporated to dryness, and the residue weighed. Each buffer plate was adjusted to a *p*H of 9–10 by addition of concentrated ammonia, washed with three 25-cc. portions of chloroform, the combined chloroform extracts dried, evaporated to dryness, and the residue weighed. The distribution pattern is shown in Fig. 1.

In a modified two-plate distribution a 6-Gm. sample was dissolved in 300 cc. of chloroform and shaken with 300 cc. of 0.5 *M* citrate buffer, *p*H 4.25, for two minutes. The chloroform layer was then shaken with two additional 300-cc. portions of buffer. A fresh 300-cc. portion of chloroform was then shaken with each of the three buffer layers in turn and this procedure was repeated with six additional 300-cc. portions of chloroform, resulting in three buffer plates and eight chloroform plates. All of the chloroform plates were combined, dried, and the chloroform removed *in vacuo*. Buffer plate 0 was made alkaline by the addition of sodium hydroxide solution, extracted with three 100-cc. portions of chloroform, the combined chloroform extracts dried and evaporated *in vacuo*. Buffer plates 1 and 2 were combined and the alkaloid removed from them in the same way as described for buffer plate 0.

By processing a total of 83 Gm. of fraction Sc, benzene soluble, in this way there was obtained 23 Gm. of material in buffer plate 0 (A); 5.5 Gm. in buffer plates 1 and 2 (B); and 43 Gm. in chloroform plates 0 through 7(C).

Partition Chromatography of (B).—Partition columns were prepared using 15 Gm. of silicic acid (Mallinckrodt, 100 mesh, chromatographic grade) and 15 cc. of 0.5 *M* citrate buffer of *p*H 5.0, in tubes of inside diameter 2.5 x 30 cm., packed in the usual way (4). A 200-mg. sample of (B) in 2 cc. of chloroform was added to the top of the packed column and, with chloroform as the eluant, a 3-cm. head was used to maintain an elution rate of one drop per second. Five-cc. fractions were collected every twelve minutes, placed in tared aluminum dishes, the chloroform allowed to evaporate, and the residues weighed (4). Chloroform was used for the first 33 fractions; thereafter the eluant was changed to chloroform-ethanol (95:5).

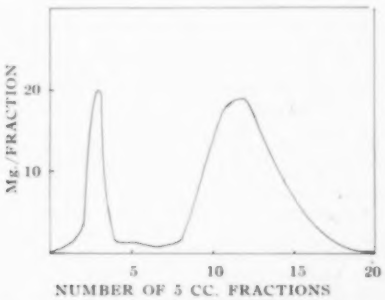


Fig. 7.—Partition chromatography of methylated vanilloylcevine with 0.5 *M* citrate buffer at *p*H 4.0 as internal phase and chloroform as eluant.

The elution pattern is shown in Fig. 2. Fractions 0–6 contained 14 mg. of material with a U.V. spectrum indicating the presence of impure veratridine. Fractions 7–22 contained 66 mg. of material which crystallized from alcohol as white needles, m. p. 210–2°, which gave no depression when mixed with an authentic sample of cevadine. Fractions 23–42 contained 83 mg. and fractions 27–39 contained 71 mg. of material which crystallized from acetone as blunt needles, m. p. 256–7° (decompn.). These recrystallized from absolute ethanol as white silky needles, m. p. 257.5–258.5° (decompn.) with previous darkening.

Anal.—Calcd. for vanilloylcevine, $C_{38}H_{49}NO_{11}$: C, 63.73; H, 7.43. Found: C, 63.50, 62.77; H,

7.29, 7.13. $[\alpha]_D^{25} = +43.6$ (c = 1.87, pyridine).

Fractions 43–52 contained 26 mg. of material which was not characterized.

Modified Countercurrent Distribution and Partition Chromatography of (A).—A 5-Gm. sample of (A) in 250 cc. of chloroform was shaken with 250 cc. of 0.5 *M* phosphate buffer, *p*H 6.75, for two minutes. The chloroform layer was then shaken with one additional 250-cc. portion of buffer. A fresh 250-cc. portion of chloroform was then shaken with each of the two buffer layers in turn. This procedure resulted in two buffer plates and two chloroform plates. Each of the buffer plates was made alkaline by the addition of concentrated ammonia, extracted with three 100-cc. portions of chloroform, the combined chloroform extracts dried and evaporated to dryness. From a total of 19.6 Gm. of (A) there was thus obtained 3.9 Gm. and 1.82 Gm., respectively, from buffer plates 0 and 1. The material in both buffer plates was indicated by U.V. spectra, to contain sabadilline (3).

The two chloroform plates were combined, dried, and evaporated to dryness to yield, from 19.6 Gm. of (A), 11.85 Gm. of alkaloidal material which was indicated, by its U.V. spectrum, to contain vanilloylcevine. A 200-mg. sample was subjected to partition chromatography, using 0.5 *M* phosphate buffer at *p*H 6.5 as internal phase and chloroform as eluant for the first 14 fractions, with 5-cc. fractions collected every ten minutes. The elution pattern is shown in Fig. 5. Fractions 5–10 contained 27 mg. of material which crystallized from acetone and was shown to be identical to the vanilloylcevine obtained from (B) above. The other alkaloidal material eluted from the column was not characterized.

Hydrolysis of Vanilloylcevine.—A sample of 660 mg. of vanilloylcevine was dissolved in 30 cc. of methanol, 20 cc. of water, and 3 cc. of 5% aqueous sodium hydroxide solution added, and the solution refluxed for thirty minutes. After cooling, the solution was adjusted to *p*H 6.0 by addition of 18% hydrochloric acid and the methanol removed *in vacuo*. The remaining solution was made slightly basic with 10% sodium hydroxide, then adjusted to *p*H 9 to 10 with concentrated ammonia, extracted with three 25-cc. portions of chloroform, the combined chloroform extracts washed with water, dried, and evaporated to dryness *in vacuo*.

The aqueous alkaline layer was acidified with 18% hydrochloric acid, extracted with three 25-cc. portions of chloroform, the combined chloroform extracts dried and evaporated to dryness to yield 25 mg. of residue. This crystallized from water as white silky needles, m. p. 205–7°, which showed no depression when mixed with an authentic sample of vanillic acid.

Anal.—Calcd. for vanillic acid, $C_8H_8O_4$: C, 57.14; H, 4.80. Found: C, 57.31; H, 4.87.

Since the U.V. spectrum of the chloroform-soluble alkamine portion indicated incomplete hydrolysis, this residue was submitted to a nine-plate countercurrent distribution to separate the alkamine from unhydrolyzed ester. Using 10-cc. portions of chloroform as the mobile phase and 10-cc. portions of 0.5 *M* phosphate buffer, *p*H 6.75, as the immobile phase, the distribution pattern shown in Fig. 6 was obtained.

Plate 0 was made alkaline by the addition of concentrated ammonia and shaken, the chloroform layer withdrawn, the aqueous layer washed with two additional 10-cc. portions of chloroform, and the combined chloroform layers evaporated to dryness. The residue (205 mg.) was dissolved in 3 cc. of chloroform and 2 cc. of ether added. Upon standing a total of 47 mg. of white prisms crystallized, m. p. 239–241°, after previous softening from 160°. Recrystallization from the same solvent yielded a product, m. p. 242–3°, the infrared spectrum of which was identical with that of cevagenine obtained from cevine by an identical hydrolysis procedure and with that reported by Stoll (5).

Methylation of Vanilloylcevine.—An ethereal solution of diazomethane prepared from 2 Gm. of N-nitrosomethylurea was added to 20 cc. of a chloroform solution of 300 mg. of vanilloylcevine which had been cooled to 5°. After sixteen hours the solvent was removed *in vacuo* and the solid residue of 247 mg. was submitted to partition chromatography (2), using 0.5 *M* citrate buffer, *p*H 4.00, as internal phase and chloroform as eluant. The distribution pattern shown in Fig. 7 is identical to that obtained for veratridine under these conditions. The material contained in fractions 8–18, melted at 150–165°, could not be crystallized, and had infrared and U.V. spectra identical with those of veratridine.

REFERENCES

(1) Poetsch, C. E., and Parks, L. M., THIS JOURNAL, **38,** 522(1949).
(2) Svoboda, G. R., and Parks, L. M., *ibid.*, **43,** 584 (1954).
(3) Poetsch, C. E., Higuchi, T., and Parks, L. M., *ibid.*, **38,** 525(1949).
(4) Henning, A. J., Higuchi, T., and Parks, L. M., *ibid.*, **40,** 168(1951).
(5) Stoll, A., and Seebeck, E., *Helv. Chim. Acta*, **35,** 1270(1952).
(6) Barton, D. H. R., and Eastham, J. F., *J. Chem. Soc.* **1953,** p. 424.
(7) Woodward, R. M., *et al.*, *Experentia*, **10,** 81(1954).
(8) Kupchan, S. M., *et al.*, *J. Am. Chem. Soc.*, **75,** 5519 (1953).
(9) Pelletier, S. W., and Jacobs, W. A., *ibid.*, **75,** 3248 (1953).
(10) Kupchan, S. M., and Lavie, D., *ibid.*, **76,** 314(1954).
(11) Marion, L., Ramsay, D. A., and Jones, R. N., *ibid.*, **74,** 270(1952).
(12) Marion, L., Ramsay, D. A., and Jones, R. N., *ibid.*, **73,** 305(1951).

A New Approach to the Chemistry of Aromatic Ammonia Spirit*

By WILLIS E. MOORE† and DAVID ABRAHAM‡

Standard reference texts state that the milky-white precipitate encountered in the preparation of Aromatic Ammonia Spirit, U.S.P., is unreacted ammonium bicarbonate due to an insufficient ammonia concentration. An examination of chemical equivalents shows that there is an excess of ammonia present. The precipitation is due to the volatile oils thrown out of solution by the lowering of the alcoholic concentration. A crystalline precipitate of normal ammonium carbonate can be produced by control of certain conditions.

DURING THE conduct of a course in inorganic pharmaceutical chemistry, it was noted that standard reference texts (1–5) contained what, upon closer examination, appeared to be misinterpretations of chemical facts concerning the chemistry of Aromatic Ammonia Spirit, U. S. P. one of the statements in question is quoted from a textbook (1).

"Upon exposure to air, official ammonium carbonate loses carbon dioxide and is converted into a white, opaque, powdery substance which is chiefly ammonium bicarbonate. If this material, instead of translucent pieces, is employed, there is not sufficient ammonium hydroxide allowed by the formula to convert the large excess of ammonium bicarbonate into normal ammonium carbonate. This fact becomes apparent only when the clear aqueous solution of normal carbonate and ammonium bicarbonate is poured into the the alcoholic solution of the oils, when the ammonium bicarbonate is thrown out of solution as an exceedingly fine precipitate that defies removal by filtration."

An examination of the chemical equivalents involved in this reaction shows that if the required 34 Gm. of Ammonium Carbonate, U. S. P., were replaced by 34 Gm. of reagent grade ammonium bicarbonate, only 7.31 Gm. of ammonia would be required to convert the primary salt into the normal salt. The 8.55 Gm. of ammonia called for by the formula represents an excess of ammonia.

An ammonia spirit prepared with totally decomposed ammonium carbonate would not, however, meet the purity rubric for total ammonia content. The spirit would be deficient in total ammonia content. This occurs because official ammonium carbonate donates an average of 31.5 per cent of ammonia to the preparation, while ammonium bicarbonate cannot donate more than 21.5 per cent (Table I).

The second point in question is the nature of the precipitate that results upon mixing the aforementioned solutions.

The exceedingly fine precipitate should be an emulsion of volatile oils precipitated by a change in alcoholic concentration. It is well known that Aromatic Ammonia Spirit, U. S. P., will precipitate oils upon the addition of a slight amount of water.

The history of Aromatic Ammonia Spirit offers a possible explanation of how the misinterpretations of the chemistry of the spirit may have occurred.

The first recognized Spiritus Salis Ammoniaci in the "London Pharmacopoeia" of 1677, was a solution of ammonia and ammonium carbonate prepared by distillation (6). In 1721, the "London Pharmacopoeia" made Spiritus Salis Volatilis Oleosus official (7). This spirit differed from its predecessor in that cinnamon, clove, mace, and lemon peel were distilled along with the required inorganics. During the distillation, a certain amount of ammonium bicarbonate sublimed and was entrained in the distillate. Due to its low solubility in the alcoholic solution (and no excess of ammonia in the boiler) the ammonium bicarbonate precipitated from the distillate (6). The explanation of the precipitate from the distillation process seems to have been carried along as the accepted interpretation, both past and present, of a precipitate by the simple solution method.

The "British Pharmacopoeia" of 1885 contained the first revised procedure to prevent the precipitation of ammonium bicarbonate by collecting the distillate in two fractions and dissolving ammonium carbonate (free from ammonium bicarbonate) in the second fraction (6). It is interesting to note that the "British Pharmacopoeia" still directs the preparation of Aromatic Ammonia Spirit in this manner.

* Received July 23, 1955, from the School of Pharmacy, The George Washington University, Washington, D. C.
† Assistant Professor of Pharmaceutical Chemistry, The George Washington University, Washington, D. C.
‡ Staff Pharmacist, Pharmaceutical Service, Clinical Center, National Institutes of Health, Bethesda, Md.

TABLE I.—ASSAYS AND TESTS FOR SOME CONSTITUENTS OF AROMATIC AMMONIA SPIRIT

Substance	Purity Rubric	Calculated, Ammonia	Found, Ammonia[a]	Found, Ammonium Carbonate[a]	Gasopoulos Test	Burgess Reaction
Ammonium carbonate U. S. P.	≥[b] 30% ≤[c] 33% Ammonia	..	30.4%	....	−	−
Ammonium bicarbonate, R. G. (Baker and Adamson)	....	21.5%	20.0%	....	−	−
Aromatic ammonia spirit U. S. P.	≥ 1.7 Gm. ≤ 2.1 Gm. Ammonia	..	1.8 Gm.	....	−	−
	≥ 3.5 Gm. ≤ 4.5 Gm. Ammonium Carbonate	..	....	3.6 Gm.	+	+
Aromatic ammonia spirit, prepared with ammonium bicarbonate, R. G.	....	..	1.9 Gm.	3.6 Gm.	+	+
Diluted ammonia solution U. S. P.	≥ 9 Gm. ≤ 10 Gm. Ammonia	..	10.0 Gm.	....	−	−

[a] Average of three determinations.

[b] Not less than.

[c] Not more than.

EXPERIMENTAL

Aromatic Ammonia Spirit, U. S. P., was prepared using recently assayed Ammonium Carbonate, U. S. P. (Table I). With the addition of the ammonium carbonate solution to the alcoholic solution of volatile oils, precipitation occurred.

After a few moments, some oily material was observed floating on the surface of the milky-appearing spirit. Since most volatile oils precipitate from alcoholic solutions upon the addition of water, the milky spirit was treated with talc and clarified by standard procedures. The talc was then extracted with alcohol and the alcoholic extract responded positively to two standard tests for volatile oils; the Gasopoulos test and the Burgess reaction (Table I). As a control, a similar precipitation occurred when an equivalent concentration of the mixed volatile oils in alcohol was precipitated by an equivalent volume of purified water. The talc from the control was extracted with alcohol and the same volume of alcoholic extract responded qualitatively the same to the two aforementioned tests.

The officially prepared, clarified spirit was assayed and found to meet official requirements (Table I) when assayed according to the official procedure (8).

An aromatic ammonia spirit was prepared by replacing the Ammonium Carbonate, U. S. P., with the chemically equivalent amount of recently assayed ammonium bicarbonate, reagent grade (Table I). In order to meet the requirements of the monograph, the required additional amount of Diluted Ammonia Solution, U. S. P., was used. This spirit was prepared in accordance with the official procedure. In a manner similar to the official spirit and to the above mentioned control, the precipitate obtained upon mixing gave qualitatively the same tests for volatile oils, and this unofficial spirit met all U. S. P. assay requirements (Table I).

It was noted that if an aqueous solution of Ammonium Carbonate, U. S. P. in ammonia solution was mixed with the alcoholic solution of the oils before the required period of standing (8), a crystalline precipitate, in addition to the oily precipitate, resulted. According to solubility data (9), this precipitate could be ammonium bicarbonate thrown out of solution by the alcoholic concentration. The nature of this crystalline precipitate was investigated.

Solutions of ammonia in water, ammonium bicarbonate in water, and the volatile oils in alcohol, in the concentrations required to prepare the official spirit, were cooled to 0°. The three solutions were mixed in respective order and filtered, with both procedures being completed within five minutes. The white, crystalline precipitate that formed was washed with portions of acetone, followed by a portion of ether. The crystals were air dried to constant weight and assayed by the procedure employed for ammonium carbonate of Aromatic Ammonia Spirit, U. S. P. by replacing a known volume of spirit with a known sample weight. Ammonium bicarbonate or normal ammonium carbonate may be determined by this assay because each cc. of 0.5 *N* sulfuric acid is equivalent to 0.04805 Gm. of normal ammonium carbonate (8). On an equivalency basis, each cc. of 0.5 *N* sulfuric acid is equivalent to 0.07906 Gm. of ammonium bicarbonate according to the method of assay. The assay results are given in Table II.

TABLE II.—ASSAYS FOR PRECIPITATES OBTAINED FROM ALCOHOLIC SOLUTIONS AT 0°

Sample Wt., Gm.	Acid used in Assay, cc.	Extrapolation 1[a]	Extrapolation 2[b]
0.6541	14.60	17.53	21.45
0.8024	18.60	18.32	22.27
0.6863	15.10	17.40	21.15
0.8659	19.49	17.79	21.62
0.6883	15.19	17.45	21.20
0.7621	16.68	17.30	21.03
	Av.	17.56 Av.	21.46

[a] Cc. of acid that would be used in assay if sample had been 0.7906 Gm. (10 milliequivalents of ammonium bicarbonate).

[b] Cc. of acid that would be used in assay if sample had been 0.9609 Gm. (10 milliequivalents of normal ammonium carbonate).

DISCUSSION

The assays (Table II) of the precipitates obtained from the alcoholic solutions indicate that the precipitate is normal ammonium carbonate, but there is a positive error present since more acid was consumed than would be expected. Theoretically, 0.96 Gm. of pure normal ammonium carbonate would require 20.0 cc., and 0.79 Gm. of pure ammonium bicarbonate would require 10.0 cc. of 0.5 *N* sulfuric acid in this method of assay. The sample weights used, when extrapolated to 0.96 Gm., would have required an average of approximately 21.5 cc. of 0.5 *N* sulfuric acid. In these crystalline precipitates, and in the spirit itself, the only logical source of basicity corresponding to 1.5 cc. of 0.5 *N* sulfuric acid would be ammonia. Therefore, it appears that this method of assay does not remove traces of ammonia produced by displacement with sodium hydroxide and heat (8). According to the method of assay, 1.5 cc. of 0.5 *N* sulfuric acid would be equivalent to 0.012 Gm. of ammonia (1.3 per cent w/w ammonia from a 0.96 Gm. sample).

Depending upon the procedure followed, Aromatic Ammonia Spirit, U. S. P., can produce two distinctly different precipitates: one, oily; the other, mixed oily-crystalline.

The positive tests for volatile oils in this spirit indicate that the fine, milky-white precipitate is the volatile oils thrown out of solution by a change in alcoholic concentration.

A reason why a freshly mixed (within five minutes) solution of ammonium bicarbonate in ammonia water should precipitate normal ammonium carbonate in an alcoholic solution may be postulated. The precipitation may be due to changes in dipolar solute-solvent bonding. In aqueous solution, ammonia reacts rapidly with ammonium bicarbonate to form normal ammonium carbonate. The ionization of the salt is rapid and the ions produced will then hydrolyze to produce carbonic acid and ammonium hydroxide.

If Pauling's concept of ammonium hydroxide (10) is accepted, a four-component equilibrium is found in its solutions; ammonia, ammonia solvated through the hydrogen bond, ammonium ion, and hydroxide ion.

When the alcoholic solution of the volatile oils is added to the aqueous solution, water and alcohol associate intimately and rapidly through dipole-dipole bonding (11). It is possible, therefore, that some ammonia-water dipole bonds (i.e., hydrogen bonds) are broken to permit the formation of water-alcohol dipole bonds. The above-mentioned four-component equilibrium is disturbed. The concentration of dissolved, but unbonded ammonia must increase.

The basicity of the ammonia liberated thusly is apparently more rapidly neutralized through coordinate covalence by the more highly acidic carbonic acid (from hydrolysis) than by the much less acidic water. Because of stoichiometric relationships, the normal carbonate is formed in preference to the primary carbonate. The normal carbonate then precipitates until such time as it can be ionized by the weak dielectric effect of the approximately 65 per cent alcoholic solution. (Approximate dielectric constant is equal to 45.)

Experimental evidence supports this postulate since the quantity of precipitate that formed rapidly redissolved and it was impossible to isolate more than 40 per cent of the theoretical yield of normal ammonium carbonate at 0°.

If this is the explanation of why the precipitate occurs upon immediate mixing, it may be inferred that no precipitate occurs after aqueous ammonia and ammonium bicarbonate have been in contact for twelve hours because solute-solvent interaction equilibria have been established. In the process of cooling, mixing and precipitation within five minutes, equilibria between the ammonium ion created by reaction, ammonia-water dipole bonds, and free ammonia must not yet be established.

SUMMARY

1. An examination of the chemical equivalents in the preparation of Aromatic Ammonia Spirit, U. S. P., shows that there is sufficient ammonia present to completely react with any excessive amount of ammonium bicarbonate in Ammonium Carbonate, U. S. P.

2. The precipitation occurring during the manufacture of Aromatic Ammonia Spirit, U. S. P. is due to volatile oils precipitated by a change in alcoholic concentration.

3. Normal ammonium carbonate can be precipitated during the manufacture of aromatic ammonia spirit under certain conditions. It is postulated that this precipitation is due to interference with dipolar solute-solvent equilibria.

4. The U. S. P. assay for normal ammonium carbonate in aromatic ammonia spirit does not remove traces of ammonia. This results in a positive error in the magnitude of 1.3 per cent w/w ammonia from a 0.96 Gm. sample of pure normal ammonium carbonate.

REFERENCES

(1) Rogers, C. H., Soine, T. O., and Wilson, C. O., "Inorganic Pharmaceutical Chemistry," 5th ed., Lea and Febiger, Philadelphia, 1952, p. 343.

(2) Arny, H. V., and Fischelis, R. P., "Principles of Pharmacy," 4th ed., W. B. Saunders Co., Philadelphia, 1937, p. 506.

(3) Davis, H., "Bentley's Textbook of Pharmaceutics," 5th ed., Bailliere, Tindall and Cox, London, 1949, p. 991.

(4) Crossen, G. E., and Goldner, K. F., "Pharmaceutical Preparations," 2nd ed., Lea and Febiger, Philadelphia, 1948, p. 113.

(5) Lyman, R. A., "American Pharmacy," Vol. 1, 3rd ed., J. B. Lippincott Co., Philadelphia, 1951, p. 389.

(6) *Pharm. J.*, **136** (4th Series, Vol. 82), 343(1936).

(7) Cook, E. F. and Martin, E. W., "Remington's Practice of Pharmacy," 9th ed., Mack Publishing Co., Easton, Pa., 1948, p. 394.

(8) "United States Pharmacopeia," 14th rev., Mack Printing Co., Easton, Pa., 1950, p. 41.

(9) Hodgman, M. S., "Handbook of Chemistry and Physics," 34th ed., Chemical Rubber Publishing Co., Cleveland, 1952–1953, p. 452.

(10) Pauling, L. C., "College Chemistry," W. H. Freeman and Co., San Francisco, 1952, p. 381.

(11) Lyman, R. A., "Pharmaceutical Compounding and Dispensing," J. B. Lippincott Co., Philadelphia, 1949, p. 159, 209.

p-Hydroxybenzoic Acid Esters as Preservatives II.

Acute and Chronic Toxicity in Dogs, Rats, and Mice*

By CAROL MATTHEWS, JOAN DAVIDSON, ELIZABETH BAUER, JAMES L. MORRISON, and ARTHUR P. RICHARDSON

The results of a study of the acute and chronic toxicity of *p*-hydroxybenzoic acid and the methyl, ethyl, propyl, and butyl esters in mice, rats, and dogs are presented. All of these compounds possess a low order of acute toxicity. The toxicity of the sodium salts appears to be a function of the length of the alkyl substituent, with the butyl ester about three times as toxic as the methyl ester. The main toxic effect is an acute myocardial depression accompanied by hypotension, but this is transient in nature and not cumulative. No evidence of blood damage or histological changes in tissues of treated animals was noted. In concentrations below 5 per cent, none of the esters produces any evidence of primary irritation when applied to the intact skin of man. From the results obtained, it may be concluded that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid may be used safely as preservatives in effective concentrations for foods and drugs.

ALTO, FIRMAN, AND RIGLER (1) have recently described the antibacterial, antifungal, and chemical properties and uses of certain esters of *p*-hydroxybenzoic acid[1] as preservatives. They have drawn attention to the present wide usefulness of these compounds and to the possibility of an even wider use. Since one of the fields in which they may serve a useful purpose is in the preservation of certain food products it became important to obtain more detailed information on their pharmacology than is now available.

This is the first of two papers describing certain aspects of the pharmacology of these agents. It will deal chiefly with acute and chronic toxicity studies in mice, rats, and dogs. Since the methyl and propyl esters have been used most widely, greater attention will be paid to these two derivatives than to the others. The second paper in the series will deal with studies on the physiological disposition of these drugs.

EXPERIMENTAL

Acute Toxicity in Mice.—Although a number of studies have been carried out on the acute toxicity of esters of *p*-hydroxybenzoic acid[2] they have served only to show that under the limited conditions used this series of compounds possess a low order of acute toxicity when administered by mouth. Experiments designed to determine their inherent toxicity are not available. It seemed important therefore to carry out additional acute studies if for no other reason than for orientation of the contemplated chronic studies.

A uniform strain of albino mice from a single source was used throughout. The drugs to be administered were prepared as a suspension of the free ester in 3% starch, propylene glycol, or olive oil. Solutions of the sodium salts were prepared by adding slightly more than one mole of sodium hydroxide to one mole of free ester. The volume of each injection was adjusted so that all animals given parenteral injections received 0.1 ml. of solution or suspension per 10 Gm. bodyweight. In the case of oral tests the large doses made it necessary to increase the volume of some of the higher doses. All animals were fasted for twelve hours prior to being used, but immediately after being injected were returned to cages with food and water. The data were analyzed by the method of Miller and Tainter (2). Table I summarizes all the data.

All four of the esters produced the same type of symptoms. Typically there was a rapid onset of ataxia, paralysis, and deep depression resembling anesthesia. Only rarely were there evidences of increased motor activity. In nonfatal doses recovery was prompt, seldom lasting beyond thirty minutes. Generally speaking deaths also occurred within a short time, usually one hour. All animals, however, were observed for a period of one week and the results reported here represent effects obtained from the entire period of observation.

The results observed with *p*-hydroxybenzoic acid deserve special mention since the results recorded with this compound at first appear somewhat confusing. Whether one uses the free acid or the sodium salt makes a remarkable difference. As the salt this substance has a remarkably low toxicity when compared with the esters. As the free acid, however, it is approximately 15 times as toxic on intraperitoneal injection as is the salt. Autopsy of some of the animals which died showed a finding which might help explain this phenomenon. Animals injected with the free acid uniformly showed considerable amount of free fluid in the peritoneal cavity, probably caused by local irritation. Such an effect was not observed with the sodium salt. Although this result was somewhat mystifying, for-

* Received January 26, 1956, from the Department of Pharmacology, Emory University, Ga.
Supported in part by a grant from Heyden Chemical Corporation.

[1] Although esters of *p*-hydroxybenzoic acid are easily described by their chemical names the methyl and propyl esters are described by U. S. P. XV as Methylparaben and Propylparaben. Available under the trademarked names of Methyl Parasept®, and Efhyl Parasept®. In this paper and in the succeeding one, all samples used were obtained from the Heyden Chemical Corporation.

[2] For a summary of earlier work see review by Neidig, C. P., and Burrell, H., *Drug and Cosmetic Ind.*, **54**, 408(1944).

TABLE I.—ACUTE TOXICITY OF PARAHYDROXYBENZOIC ACID AND ITS ESTERS IN MICE

Compound	Oral								Intravenous		Intraperitoneal			
	Free Acid		Na Salt		Propylene Glycol		Olive Oil		Na Salt		Free Acid		Na Salt	
	PD_{50}[a]	LD_{50}[b]	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}
p-Hydroxybenzoic acid	..	..	No paralysis	2200 ±89.4	..	..	..	..	1050 ±60.1	1200 ±58.9	No paralysis	210 ±18.8	No paralysis	3400 ±155.2
Methyl *p*-hydroxybenzoate	>8000	>8000	1300 ±123.4	2000 ±141.2	>8000	>8000	Approx. 8000	Approx. 8000	80 ±9.7	170 ±8.4	370 ±36.3	960 ±70.5	460 ±22.1	760 ±34.7
Ethyl *p*-hydroxybenzoate	..	..	..	Approx. 2500	..	..	..	..	..	..	..	..	280 ±50	520 ±60.0
Propyl *p*-hydroxybenzoate	No paralysis	>8000	No paralysis	3700 ±189.5	>8000	>8000	>8000	>8000	54 ±5.0	180 ±3.7	460 ±30.3	640 ±46.1	490 ±20.9	490 ±20.9
Butyl *p*-hydroxybenzoate	..	..	..	Approx. 950	..	..	..	..	..	..	..	..	200 ±65	230 ±24

[a] PD_{50} = Dose required to paralyze 50% of animals. [b] LD_{50} = Dose required to kill 50% of animals. All figures represent mg./Kg.

tunately no such differential effect was observed with any of the esters so that although it is difficult to compare the results obtained on the esters with *p*-hydroxybenzoic acid the data obtained do permit certain conclusions as to the relative acute toxicity of the four esters. In each instance where they were compared under the same conditions there was a consistent relationship between the length of the alkyl chain of the alcohol and order of toxicity. Thus it is quite apparent that in all instances the methyl ester was least and the butyl ester most toxic. This was illustrated in the intraperitoneal toxicity tests carried out with the sodium salts. In this particular series of experiments butyl *p*-hydroxybenzoate is approximately three times as toxic as the methyl ester. As will be pointed out later this difference in toxicity is correlated with the speed of hydrolysis of the various esters.

The oral tests do not contribute a great deal to the general understanding of the action of this group of compounds since the doses required to produce toxic effects are so large as to make it difficult to obtain an entirely satisfactory dosage-response curve. Since these compounds are more soluble in alcohols and fatty substances such as olive oil it seemed important to determine whether absorption from the gastrointestinal tract might not be speeded up by incorporating them in such a vehicle. The data in Table I clearly indicate a lack of effect of either olive oil or propylene glycol on absorption from the gut. On the other hand the use of sodium salts did appear to enhance absorption since the fatal doses for these preparations were significantly lower than when a suspension of the free esters was used. It is important to point out here that the great difference in response between oral and parenteral administration cannot be explained on a basis of incomplete absorption. Results to be reported in a succeeding paper (3) of this series showed excellent recoveries from the urine of animals after oral administration. The low oral toxicity, therefore, is to be explained by the fact that *p*-hydroxybenzoates have a rapid but brief action due to rapid metabolic alteration. Therefore rate of absorption is an important factor in explaining the wide variation of oral and parenteral toxicity.

Acute Toxicity in Dogs.—In order to more accurately determine the mechanism of acute death caused by this series of compounds, unanesthetized dogs and dogs anesthetized with 80 mg./Kg. of chloralose, or 25 mg./Kg. of pentobarbital were used for more detailed studies. Aside from some depression observed in unanesthetized animals the effects observed were the same regardless of preoperative preparation. One femoral artery was cannulated and connected to an Anderson glass capsule for recording of blood pressure. A femoral vein was cannulated and connected to a reservoir system to permit injection of drugs. In some experiments a glass cannula was inserted into a jugular vein to permit recording of venous pressure a few centimeters from the right auricle. A standard Sanborn Visocardiette was used for recording of electrocardiograms. Sodium salts of each of the compounds were prepared as previously described. Standard responses to intravenous doses of epinephrine, acetylcholine, and histamine were obtained as controls to test the function of the cardiovascular and autonomic nervous system. Following control

observations, gradually increasing doses of each of the compounds were injected and effect on the cardiovascular system, and autonomic nervous system determined.

The only important action observed was a precipitous but brief fall in blood pressure associated with a rise in jugular venous pressure as illustrated by Fig. 1. In these acute experiments, death was

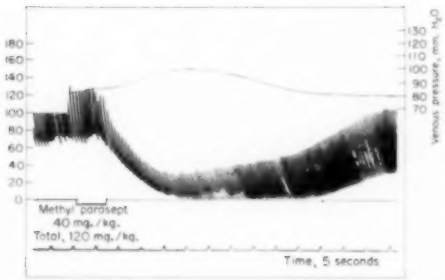


Fig. 1.—Effect of intravenous injection of 40 mg./Kg. of methyl parahydroxybenzoate. Dog anesthetized with chloralose. Upper line venous pressure at the level of the right auricle.

associated with the hypotensive action, and since cardiovascular effects were brief, it was not unexpected to find an important relationship between the action on the cardiovascular system and the rate of injection. For example in the case of the propyl ester the fatal dose is of the order of 85 mg. per Kg. when administered at the rate of 20 mg./Kg./minute, whereas when the same drug was administered at the rate of 5 mg./Kg./minute, the fatal dose is of the order of 600 mg./Kg. Table II summarizes the data obtained with the methyl and propyl ester on this point. Obviously then in order to compare the relative action of the compounds under study the rate of injection was carefully controlled. Arbitrarily, the effect on blood pressure of single doses injected within a ten-second period was determined. Figure 2 illustrates a typical record of the type of experiment carried out. The fall in systolic pressure was measured and dosage-response curves con-

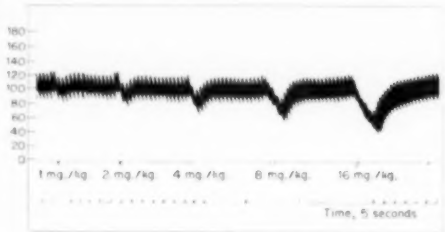


Fig. 2.—Effect of increasing intravenous injections of propyl parahydroxybenzoate on the blood pressure of dogs.

structed as illustrated in Fig. 3. To simplify presentation of the data the dose necessary to produce 35 mm. fall in pressure was determined. As in the case of acute toxicity studies in mice there is a correlation between the length of the alcohol alkyl chain and hypotensive effect. The "35-mm. hypotensive dose" being as follows: *p*-hydroxybenzoic acid 40 mg./Kg., methyl ester 16 mg./Kg., ethyl 16 mg./Kg., propyl ester 8 mg./Kg., and butyl ester 8 mg./Kg. Since the method of preparing the sodium salts utilized a slight excess of alkali and gave solutions of *p*H 9–10 it was important to rule out the possibility that these cardiovascular effects might have been due to alkali. This was controlled by a study of solutions prepared with sodium bicarbonate instead of sodium hydroxide. The *p*H of such solutions was 7.5–8.0. These nearly neutral solutions produced the same depressor effect as the more alkaline solutions. In addition the injection of sodium hydroxide solutions in doses several times those used in the above studies were without depressor effect.

The mechanism of the hypotensive action appears to be depression of the heart since it was accompanied by significant electrocardiographic changes. Figure 4 illustrates a typical set of tracings obtained in the course of blood pressure change shown in Fig. 1. At the nadir of the blood pressure response there was a widening of the QRS complex with a decrease in potential and inversion of the *T* wave. These changes are interpreted as being evidence of

TABLE II.—EFFECT OF RATE OF INJECTION ON INTRAVENOUS FATAL DOSE OF ESTERS OF PARAHYDROXYBENZOIC ACID IN DOGS

Rate of Injection									
5 mg./Kg./min.		10 mg./Kg./min.		15 mg./Kg./min.		20 mg./Kg./min.		40 mg./Kg./min.	
Total dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect
						Methyl *p*-Hydroxybenzoate			
						360	Surv.	80	Died
						940	Died	320	Died
						1140	Surv.	600	Died
						1400	Died	920	Surv.
						..	..	1100	Surv.
		Propyl *p*-Hydroxybenzoate							
470	Surv.	600	Died	210	Died	120	Died		
870	Surv.	720	Died	150	Died	80	Died		
400	Surv.	434	Surv.	60	Died	80	Died		
..	..	1080	Surv.	75	Died	80	Died		
..	..	..	..	100	Died	80	Died		
..	..	..	..	495	Surv.	100	Died		

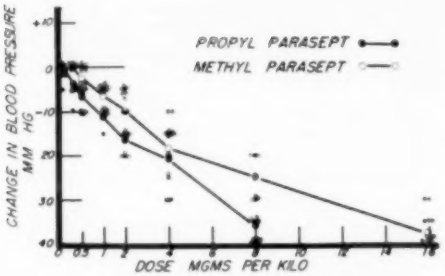


Fig. 3.—Summary of effects of single doses of methyl and propyl parahydroxybenzoates on the blood pressure of dogs. Small circles represent individual experiments. Large circles represent mean values.

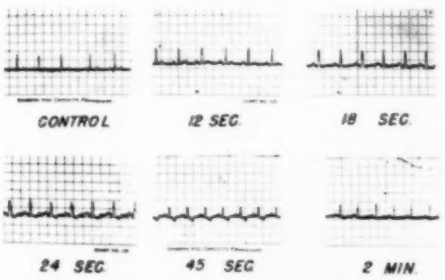


Fig. 4.—Effect of methyl parahydroxybenzoate on the electrocardiogram of the dog. Lead II taken in the course of experiment illustrated by Fig. 1.

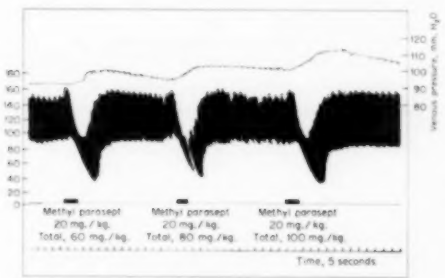


Fig. 5.—Effect of repeated doses of methyl parahydroxybenzoate on the blood pressure. Note lack of cumulative effect.

depression of the myocardium and are not unlike the effects produced by procaine and procaine amide. As in the case of the blood pressure fall, the electrocardiographic changes are quickly reversible and transient. As is shown in Fig. 5 there is little indication of cumulative effect. Associated with the hypotensive action there was uniformly a marked rise in right auricular pressure which is also indicative of cardiac depression.

Of some interest were the negative effects of the *p*-hydroxybenzoates on the autonomic nervous system. Even at near fatal doses there was no change in the response of the animals to epinephrine, acetylcholine, or histamine. Also full doses of atropine (1.0 mg./Kg.) or section of the vagi had no significant effect on the fall in blood pressure. Since metabolic studies indicated that all of the esters are rapidly hydrolyzed in the body it was of interest to find that doses of physostigmine sufficient to produce skeletal tremors (0.2 mg./Kg.) had no effect on the cardiovascular action; it seems fair to assume therefore that choline esterases are not responsible for the metabolic alteration of the compounds.

Chronic Toxicity in White Rats.—Since the indications at the outset of these studies were that the propyl and methyl esters were particularly apt to be used as food preservatives, extended chronic studies were carried out on these two compounds. Preliminary metabolic studies showed that absorption from the gastrointestinal tract was essentially complete (3) hence it seemed safe to study the chronic toxicity in animals in which the drug was incorporated in the diet. Purina Dog Chow was used as a diet throughout the entire experiment. Checkers were first ground roughly in a flour mill, and after the addition of an appropriate amount of drug the mixture was shaken and run through a flour mill four or five times to insure complete mixing. On the last run through the mill the food was ground as finely as the apparatus would permit. As a test to insure that the drug had been evenly and completely distributed in the diet, a test sample of dog checkers was ground with a known quantity of sulfanilamide and assayed chemically. Assay of a sulfanilamide diet before and after feeding showed that animals were unable to select food instead of drug and therefore it was assumed that this method of preparing diets insured continuous and even intake of drug.

Twenty-four weanling albino rats from a strain originally obtained from the Wistar Institute[2] were equally divided between males and females at each dosage level. Animals were selected at random from a large batch of weanling rats. At the start animals were placed in cages in groups of four, but as they matured they were separated so that there were two animals to a cage. Drug diet was placed in containers which prevented wasting by the rats; free access was permitted to both food and water. Food intake and weight of animals was determined every two weeks. Control animals were fed a similar diet of powdered dog checkers but without drug.

Three series of experiments as outlined above were carried out. In the first, a group of animals was given diets containing 2% of propyl and methyl esters of *p*-hydroxybenzoic acid, and compared with animals given no drug. After forty weeks on this diet, it was apparent that this concentration was having no effect on the growth curve and Series II was set up in similar fashion but containing 2% and 8% of each drug. In both Series I and II animals were treated for a period of ninety-six weeks. As these series of experiments were reaching completion it seemed advisable to also run a short term chronic toxicity on the ethyl and butyl esters.

[2] All rats used in these studies were obtained from Mr. Winfrey Winn, Department of Physiology of Emory University, who has maintained this colony unbroken for at least the past ten years.

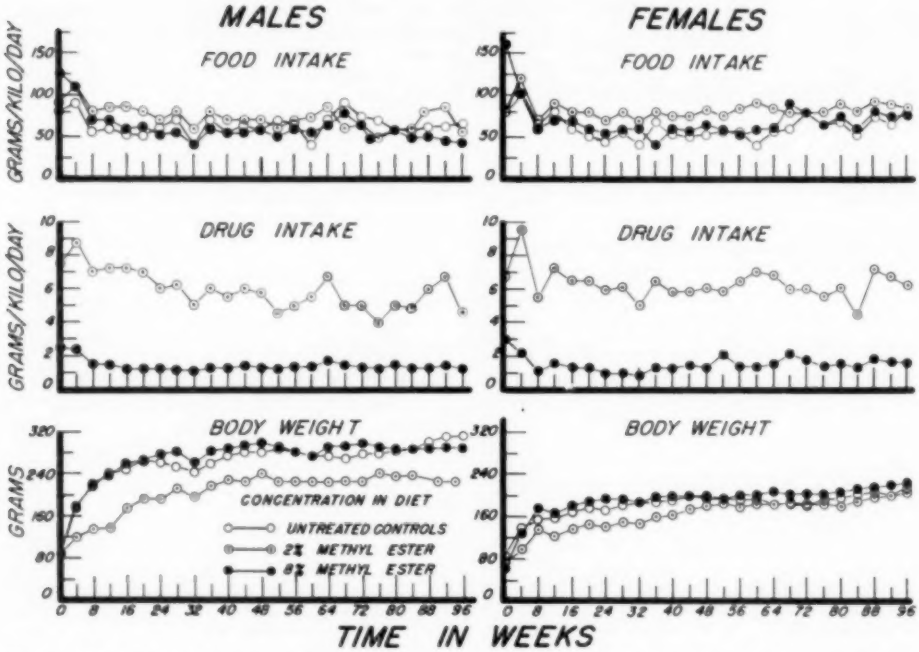


Fig. 6.—The effect of incorporation of methyl parahydroxybenzoate in the diet of weanling white rats. Each curve represents mean of 12 animals.

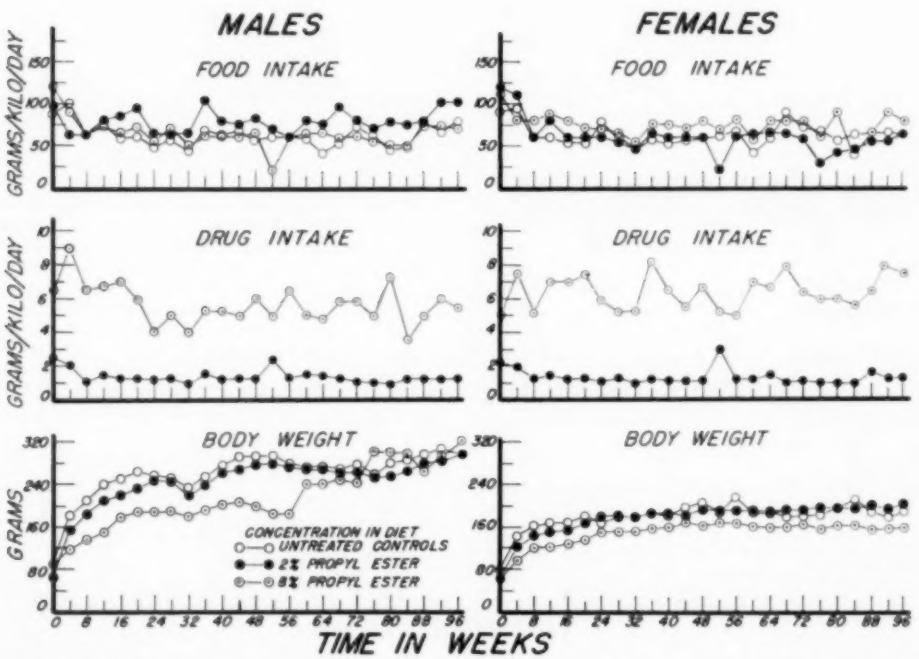


Fig. 7.—The effect of incorporation of propyl parahydroxybenzoate in the diet of weanling white rats. Each curve represents mean of 12 animals.

Therefore Series III was set up with rats on 2% and 8% of these two esters but in which the test period was allowed to run for only twelve weeks. At the conclusion of each experiment all animals, control and experimental, were sacrificed and appropriate tissues removed for pathological study. All animals dying in the course of the experiment were autopsied and tissues removed and fixed for later study by a pathologist.

Since the results obtained in Series II were similar but more extensive than Series I, only the results with the former are presented in detail here. Figures 6, 7, and Table III summarize the results obtained in Series II. Animals receiving diets 8% of both the propyl and methyl esters all showed a slower rate of gain in weight than did controls. This difference is much more striking in the early part of the experiment. In fact, as the end of the test was approached these differences tended to disappear. This effect was more striking in the case of the males than the females. On animals receiving 2% of these drugs, however, it was impossible to differentiate treated animals from untreated animals.

Series III was carried out in the same manner as the first two series except that younger rats were used and ethyl and butyl esters were incorporated in the diet. Essentially the same results were obtained with the ethyl ester as with the methyl and propyl esters, namely concentrations of 2% were without effect, but 8% showed not only a much slower rate of growth, but also signs of toxicity characterized by depression, decreased motor activity, and deaths within the first few weeks of beginning the experiment. In fact all the males on the 8% diet of butyl ester died before the end of the twelve-week period of observation. Although these symptoms were less striking with females on this same diet, this group also had many early deaths and depression.

Since it has already been pointed out that the most striking changes were observed within the first part of the experiment, Table III has been constructed so as to show a rough comparison of the effects produced by all four drugs at twelve weeks. The results clearly show the lack of effect of diets containing 2% of any of the four compounds whereas in each instance a definite decrease in weight was observed in all groups fed the 8% diets.

Food intake and hence drug intake remained fairly constant throughout the course of the various feeding experiments, nor were there significant differences between the various esters. Drug intakes for animals on the 2% diets averaged from 0.9 to 1.2 Gm./Kg. body weight per day. While the drug intake of rats on the 8% diets averaged from 5.5 to 5.9 Gm./Kg./day. If it is valid to transfer results from rat experiments to other species it would be safe to assume that on chronic studies the first signs of toxicity would not appear until at least a drug intake of 3 Gm./Kg./day had been reached.

Chronic Toxicity in Dogs.—A group of eleven young healthy mongrel pups were taken from their mothers after weaning and divided into five groups as shown in Table IV. Each dog was housed in an individual cage but permitted to run in an enclosed area from time to time. All animals were fed Purina Dog Chow *ad libitum* and in addition were given biweekly supplements of meat scraps. After they had become adjusted to the diet and seemed to be gaining weight, oral treatment with the methyl and propyl *p*-hydroxybenzoates was started. The drugs were put up in gelatin capsules of convenient size so that proper dosage could be readily administered. Treatment was given six days weekly (Sunday excluded). No toxic symptoms were observed in any of the animals. One control died 195 days after the beginning of the experiment apparently of pneumonia. The weights of animals before and after treatment are given in Table IV and for the most part showed respectable increases. Since these animals were mongrels of varying antecedents it was to be expected that final weights would vary greatly. Of most importance however was the fact that at the end of the experiment all animals appeared to be in excellent condition as far as general appearance was concerned. Of secondary interest it is worth noting that one of the females receiving 0.5 Gm./Kg./day of methyl ester was mated toward the end of the experiment and delivered a litter of healthy pups which were later raised in the laboratory.

Approximately one month before the end of the experiment all animals receiving 1.0 Gm./Kg. of either ester were studied for possible cumulation of compound in the blood. This was done in the following manner: Just prior to the regular daily dosing a control blood sample was taken and analyzed according to methods described elsewhere (3). After the usual dose had been administered blood samples were taken over the next twenty-four hours and analyzed for drug and metabolic end products. The results of this study are summarized in Table V. It is to be noted that at zero time

TABLE III.—A COMPARISON OF THE EFFECT OF ESTERS OF PARAHYDROXYBENZOIC ACID ON THE GROWTH CURVE OF WEANLING ALBINO RATS. EACH GROUP REPRESENTS THE MEAN OF 12 RATS. PERIOD OF TEST 12 WEEKS

Drug Diet	Sex	Mean Wt. of Rats Before Treatment, Gm.	Wt. After Treatment, Gm.	Gain in Wt., %
	Series II			
Control	M	90	240	144
2% Methyl ester	M	90	240	144
8% Methyl ester	M	93	141	52
2% Propyl ester	M	71	203	188
8% Propyl ester	M	87	144	63
Control	F	78	170	119
2% Methyl ester	F	69	164	136
8% Methyl ester	F	75	120	60
2% Propyl ester	F	65	160	146
8% Propyl ester	F	81	120	48
	Series III			
Control	M	38	263	610
2% Ethyl ester	M	39	264	601
8% Ethyl ester	M	30	164	430
2% Butyl ester	M	32	240	654
8% Butyl ester	M	31	Died	..
Control	F	31	198	538
2% Ethyl ester	F	33	180	440
8% Ethyl ester	F	33	151	350
2% Butyl ester	F	34	210	510
8% Butyl ester	F	34	115	235

(twenty-four hours following the previous treatment) a small amount of assayable material was still present in the blood, chiefly as conjugated end products. However the plasma concentration-time curve resembled closely that of dogs not previously treated. In addition urine was quantitatively collected over same period of time and it is highly significant that in the case of both compounds between 96% and 100% of the daily administered dose was accounted for in the urine, chiefly in the form of conjugated end products. Both the urine and plasma studies therefore furnish strong evidence of the lack of cumulation of either the methyl or propyl *p*-hydroxybenzoate when administered at the level of 1.0 Gm./Kg./day for one year.

Near the conclusion of the period of observation, complete blood counts and urine examinations were made on all dogs. Details of these experiments are omitted since the findings were entirely normal. At the end of the experiment all dogs were killed by the injection of air and turned over to the pathologist for careful autopsy and histological study of selected tissues.

Pathological Studies on Tissues.—As described above all animals dying in the course of chronic toxicity studies on rats and dogs were autopsied and selected tissues preserved for histological examination. At the conclusion of the chronic studies all animals were sacrificed and tissues selected for study. The various specimens were labeled with a code number and given to Dr. Abner Golden of the Pathology Department of Emory University School of Medicine. Particular attention was paid to kidney, liver, heart, lung, spleen, and pancreas. According to Dr. Golden's report, tissues from all rats surviving the chronic tests were entirely normal. The only significant findings were seen in the case of rats dying in the course of experiments; in all such animals there was extensive consolidation of the lung and pneumonia, and in this respect there was no difference between the control and treated animals.

Inasmuch as an important suggested use of esters of *p*-hydroxybenzoic acid is in cosmetics it is important to know whether these agents possess any local irritative property and whether they may produce sensitization. Studies on this point have been made by other investigators and a summary of their unpublished results is included below.

Allergic Reactions to Esters of *p*-Hydroxybenzoic Acid in Guinea Pigs.[4]—Guinea pigs were injected intracutaneously with a solution of the ester at regular intervals for three and one-half weeks, then allowed to rest for two weeks. During this period a sensitivity to the compound, if it existed, would have been built up. At the end of the period a challenging dose was given, and the reaction to it was read after twenty-four hours. A difference in the area of irritation between the first and challenging doses would have been a positive reaction.

The procedure used was the one suggested by H. Draize (4). This is a slight modification of the method outlined by Landsteiner and Jacobs.

Forty white male guinea pigs weighing 300 to 450 Gm., subsisting on a Purina rabbit pellet ration supplemented with greens (kale and lettuce), were identified and hair was removed from back and flanks by close clipping. The pigs were divided into four groups, ten for each ester.

Solutions of 0.1% in physiological saline were prepared, the methyl and ethyl esters used as such, and the propyl and butyl esters as the sodium salts made by dissolving the esters in the calculated amount of 5% sodium hydroxide solution, then diluting. The solutions were injected intracutaneously using a 27-gauge hypodermic needle. The animals were injected three times weekly at random in an area of the back and upper flanks four centimeters square. A total of ten injections was given. The first injection consisted of 0.05 ml.; the remaining ones were 0.1 ml. No reaction was noted twenty-four hours after the first injection.

Two weeks after the tenth injection, a retest injection was made using 0.05 ml. of a freshly prepared solution. The retest injection was made in an area just below the sites of the sensitizing injection. Twenty-four hours later the guinea pigs were closely observed for a reaction; there was none.

Primary Irritation and Sensitization in Man[5]

Primary Irritative Effect.—The methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid were dissolved in propylene glycol in increasing concentrations, and applied to the skin of 50 human subjects. It was found that the following concen-

TABLE IV.—SUMMARY OF CHRONIC ORAL TOXICITY IN DOGS

Methyl Parasept® Orally						Propyl Parasept® Orally					
1 Gm./Kg. Daily			0.5 Gm./Kg. Daily			1 Gm./Kg. Daily			0.5 Gm./Kg. Daily		
No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.
378	3.6	9	318	3.5	8.8	394	4.5	6	394	4.1	5
422	3.6	12	331	8.6	9.4	313	4.7	4.5	...	...	...
394	4.5	5.5	...	...	...	384	6.4	12	...	...	...

Control

No. of Days	Initial Wt. Kg.	Final Wt., Kg.
422	2.3	12.3
195	4.6	7.5

[4] By J. Marrus and W. M. Ziegler, Heyden Chemical Corporation, Princeton, N. J.

[5] By Herman Shelanski, Industrial Toxicology Laboratories, Philadelphia, Pa.

TABLE V.—PLASMA CONCENTRATION—TIME CURVES OF DOGS RECEIVING 1.0 GM./KG. OF METHYL AND PROPYL PARAHYDROXYBENZOATE DAILY FOR APPROXIMATELY 350 DAYS. TESTS STARTED 24 HOURS AFTER LAST DOSE

	Plasma Concentration mcg./ml.		
Time	Free Parasept®	Para-hydroxy-benzoic Acid	Total Material
0	0.5	8.0[a]	54.0
15 Min.	0	23.0[a]	93.0
1 Hr.	4.0	56.0[a]	261.0
2 Hr.	14.0	197.0[a]	313.0
4 Hr.	0	492.0[a]	655.0
6 Hr.	0	450.0[a]	591.0
24 Hr.	0	27.0[a]	43.0
0	0	10.0[b]	47.0
15 Min.	0.3	33.0[b]	55.0
1 Hr.	0	39.0[b]	122.0
2 Hr.	0	100.0[b]	315.0
4 Hr.	8	91.0[b]	226.0
6 Hr.	1	87.0[b]	265.0
24 Hr.	0	0.0[b]	17.0

[a] Methyl Parasept® (mean of 2 dogs).
[b] Propyl Parasept® (mean of 3 dogs).

trations produced no irritation when applied during a period from four to eight hours every other day and a total of ten applications; methyl *p*-hydroxybenzoate 5%, ethyl *p*-hydroxybenzoate 7%, propyl *p*-hydroxybenzoate 12%, and butyl *p*-hydroxybenzoate 5%. Higher concentrations than this produced some evidence of irritation.

Repeated Insult Method.—In 50 unselected subjects (25 males, 25 females) each of the esters was applied in the maximum tolerated concentration as described above. At the end of this period three weeks was allowed to elapse during which time there was no contact with the drugs. At this point each drug was reapplied for a period of from 24 to 48 hours in the same concentration which was used on original contact. In no instance was there evidence of development of sensitization.

SUMMARY

A study has been made of the acute and chronic toxicity of *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, and butyl esters in mice, rats, and dogs.

1. Each of these compounds has a low order of acute toxicity, which in the form of the sodium salt increases with the length of the alkyl ester, the butyl ester being about three times as toxic as toxic as the methyl ester.

2. In the case of the esters the chief toxic effect is an acute myocardial depression with hypotension. This effect is transient in nature and is not cumulative.

3. Rats fed 0.9 to 1.2 Gm./Kg./day of the four esters showed no effect even when treated as long as ninety-six weeks. Rats given 5.5 to 5.9 Gm/Kg./day showed a definite depression of rate of growth particularly during the first twelve weeks of feeding. At this higher dosage the butyl ester was definitely more toxic and killed all animals within the first twelve weeks.

4. Dogs fed 1.0 Gm./Kg./day for one year showed no evidence of toxicity.

5. There was no evidence of blood damage or histological changes in kidney, liver, heart, lung, spleen, and pancreas in any of the treated animals.

6. In concentrations up to 5 per cent, none of the esters produce evidence of primary irritation when applied to the intact skin of man. Repeated applications do not produce sensitization.

7. It would appear that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid may be used safely as food and drug preservatives in concentrations which have been recommended.

REFERENCES

(1) Aalto, T. R., Firman, M. C., and Rigler, N. E., THIS JOURNAL, **42**, 449(1953).
(2) Miller, L., and Tainter, M. L., *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1949).
(3) Jones, P. S., Thigpen, D., Morrison, J. L., and Richardson, A. P., THIS JOURNAL, **45**, 268(1956).
(4) Lehman, A. J., *et al.*, *Food, Drug, Cosmetic Law Quart.* **4**, 412(1949).

p-Hydroxybenzoic Acid Esters as Preservatives III.

The Physiological Disposition of *p*-Hydroxybenzoic Acid and Its Esters*

By PASSIE S. JONES, DORIS THIGPEN, JAMES L. MORRISON, and ARTHUR P. RICHARDSON

Free *p*-hydroxybenzoic acid and its metabolic products in which the phenolic group has not been destroyed have been determined in tissues and biological fluids of drugs, tablets, and man following the ingestion of the acid and its esters. Recoveries from the urine of animals, regardless of the method of administration, range between 50 and 95 per cent except in the case of butyl esters where the recoveries were approximately 40 per cent. High plasma levels and urinary output of free and conjugated *p*-hydroxybenzoic acid in dogs indicate that hydrolysis of the ester linkage and metabolic conjugation constitute the chief paths of alteration for all of the esters studied except the butyl derivative. Topical application of ointments containing 10 per cent methyl and 10 per cent propyl *p*-hydroxybenzoate to the shaved backs of white rabbits produced no irritation during forty-eight hours of contact. Under this condition no drug concentration was detected in the kidneys.

THE INCREASING interest in and use of esters of *p*-hydroxybenzoic acid as food, cosmetic, and drug preservatives has created the need for detailed information as to the absorption, distribution, and disposition of this series of compounds. This paper describes certain chemical methods which have been found useful for such studies and results obtained in dogs, rabbits, and man.

A quantitative method of determination of these substances in tissues and biological fluids was devised by utilizing the ability of phenols to couple with 4-amino-antipyrine (1). Under alkaline oxidizing conditions, the reagent combines with the phenolic substances *para* to its hydroxy group to form a highly colored compound. In order for this reaction to take place the phenolic group must be free and the position *para* to it unsubstituted. However, the presence of a free carboxylic acid group in the *para* position does not prohibit coupling since under the conditions used this group is removed by decarboxylation.

It was felt that this method was particularly suited to the analysis of tissues and biological fluids for *p*-hydroxybenzoic acid (PHBA) and its methyl, ethyl, propyl, and butyl esters, for if during the course of metabolism of the esters they should appear in the tissue or fluid simultaneously with the free acid, they would be distinguishable by the restrictive conditions necessary for coupling with amino-antipyrine. Hydroxybenzoates, with ester groups *para* to the hydroxy group, would necessitate hydrolysis before coupling could take place, and thus PHBA could be determined in their presence without interference. The esters, on the other hand, could be analyzed for in the presence of PHBA by making use of differential extraction since before hydrolysis their acidity, and consequently their solubility in sodium bicarbonate, differs markedly from PHBA.

From past studies with similar compounds, it was felt that further detoxication of the esters would be possible in the body (2). The phenolic group of the hydroxybenzoates could link with sulfate and glucuronic acid, and if the ester were first degraded to PHBA, the acid group would be available for glucuronic acid and glycine conjugation. Any of these conjugation products would require hydrolysis before color development could take place, and they could be in turn distinguished from the esters due to the bicarbonate solubility of the conjugates.

Simple hydrolysis of the tissue or fluid sample under study would reduce the esters and their metabolic products to PHBA, and in such a way the total material present could also be determined.

EXPERIMENTAL

Procedure.—The procedures as finally adopted for use are as follows: A buffer of pH 10–11 was prepared for the medium in which the coupling reaction takes place in order to insure maximum, stable color development. It was prepared as follows: 43.9 cc. of 0.1 *N* NaOH + 50.0 cc. of 0.1 *M* H_3BO_3 diluted to 100 cc.

Determination of PHBA in Water, Urine, and Plasma.—One cc. of plasma or 1 cc. of urine diluted 1:10, 1 cc. of 6 *N* H_2SO_4, and 7 cc. of absolute ether were shaken mechanically for six minutes in a glass-stoppered centrifuge tube. The mixture was then

* Received January 26, 1956, from Department of Pharmacology, Emory University, Ga.
Supported in part by a grant from Heyden Chemical Corporation.

centrifuged for one minute at 1500 r. p. m., and a 4-cc. aliquot of the supernatant ether solution was transferred to a test tube and the ether removed by evaporation from a warm water bath. Four cubic centimeters of the buffer were added to the tube and the solution heated for five minutes at 90–100° to insure complete solution of the residue. Upon cooling to room temperature, 1 cc. of amino-antipyridine reagent (1.36 Gm./liter) and 0.5 cc. of potassium ferricyanide solution (8.67 Gm. of $K_3Fe(CN)_6$ and 1.8 cc. of concentrated NH_4OH/L.) were added to the tube. The resulting colored solution was shaken by hand for a few seconds and the transmittance was determined at 490 mμ on the Beckman spectrophotometer. Color develops immediately upon shaking and remains stable for at least an hour.

Determination of *p*-Hydroxybenzoates in Water, Urine, and Plasma.—One cubic centimeter of plasma or urine, 1 cc. of saturated $NaHCO_3$, and 7 cc. of absolute ether were mechanically shaken for six minutes in a glass-stoppered centrifuge tube. The mixture was then centrifuged for one minute at 1500 r. p. m., and a 5-cc. aliquot of the ether phase was evaporated in a glass-stoppered centrifuge tube from a warm water bath. One cubic centimeter of water and 1 cc. of 6 *N* H_2SO_4 were added to the residue and the tube heated for two hours in a boiling water bath. It had been previously determined that further hydrolysis did not take place after 2 hours. Upon cooling, 7 cc. of absolute ether was added to the tube, and the extraction was carried out by mechanical shaking for six minutes. The remainder of the procedure was identical with the PHBA determination. Calibration curves were run for all esters using this procedure.

Determination of Total Material (PHBA + *p*-Hydroxybenzoates + Metabolic Conjugates) in Water, Plasma, and Urine.—This procedure is identical with the PHBA determination except that before the addition of the ether, the acidic phase was heated for two hours in a boiling water bath.

These methods are sensitive to 0.3–0.5 γ/cc. except in those urine determinations where dilution of 1:10 is necessary to remove interfering blank color. In those cases the sensitivity is 3–5 γ/cc. Complete standard curves were run with water, plasma, and urine standards carried through all the steps of the procedure, including extraction; all these curves were identical. No standard curves were available for the "Total" material, but values were read from the PHBA curve. By correcting for the aliquots, 75–85% of the added material may be accounted for in the procedures.

Determination of PHBA, *p*-Hydroxybenzoates and "Total" Material in Tissues.—Satisfactory analyses for these compounds have been made in liver, spleen, muscle, kidney, brain, and pancreas. The organ under study was cut up and added to an equal quantity by weight of saline. After preliminary mixing in a Waring Blendor, the resulting suspension was ground in a tissue grinder until it might be drawn through a syringe. One cubic centimeter of this mixture was used for analysis. The procedures for the determination of PHBA, *p*-hydroxybenzoates, and "Total" material in tissues are identical with those in plasma except that the ether aliquot was shaken mechanically in a glass-stoppered centrifuge tube for six minutes with 4 cc. of the buffer. The mixture was then centrifuged for 1 minute at 1500 r. p. m. and the supernatant ether phase removed by suction. Color was then developed in the buffer solution. This double extraction was found necessary to remove blank interference.

Standard curves were run in all the tissues with a sensitivity of approximately 1γ/cc. Recoveries were of the same range as those from water, urine, and plasma.

Glucuronic acid determinations were made whenever possible to reveal the extent of conjugation with this compound. The method of Maughan, Browne, and Evelyn (3) was found satisfactory for our use. Specific analysis for the other conjugates was omitted for simplicity; however, the quantity of these metabolites may be determined by subtraction of the Parasept®, free PHBA, and glucuronic acid conjugate fractions from the "Total" material.

ABSORPTION AND EXCRETION

In order to understand more fully how the body handles these drugs, a comparison was made of oral and intravenous administration of the esters and PHBA. Intravenous injections of 50 mg./Kg. of PHBA, methyl, ethyl, propyl, and butyl *p*-hydroxybenzoates were each given to a series of three or more fasted dogs, and blood and urine samples were taken at fixed intervals following the injection until levels fell to zero. Determinations of the compound and its various metabolic products were then made on the samples. A similar study was carried out in which 1.0 Gm./Kg. of the drugs was given orally in capsules to the dogs. Summaries of these experiments are tabulated in Table I, Fig. 1 and 2.

Excellent recoveries of "Total" material were noted from the urine of dogs having received intravenous and oral doses of PHBA, methyl ester, ethyl ester, and propyl ester. These ranged from 60–95% indicating that the major portion of the drug as it is metabolized and excreted by the body retains its phenolic structure. Most of the material is excreted by six hours after injected doses whereas appreciable amounts are still detected in the urine thirty hours after oral ingestion with the greater part of the drug being excreted between six and twenty-four hours. These results, along with data obtained from the plasma concentrations after oral feeding, seem to indicate that absorption is essentially complete.

Recoveries of "Total" material after oral and intravenous doses of the butyl ester were only 48% and 40%, respectively. It was felt that this might indicate a relatively less effective hydrolysis of the butyl ester by the body which would allow other degradation processes such as oxidation and reduction to take place, thus, destroying the structure necessary for coupling with amino-antipyrine. This difference in metabolic handling is noticed particularly following intravenous injections where an extremely rapid introduction of the drug into the body takes place.

Detoxication mechanisms seem to proceed efficiently immediately after ingestion of the drugs. The concentration of the *p*-hydroxybenzoates in the plasma only rarely surpasses the lower limit of sensitivity of the determinations even immediately

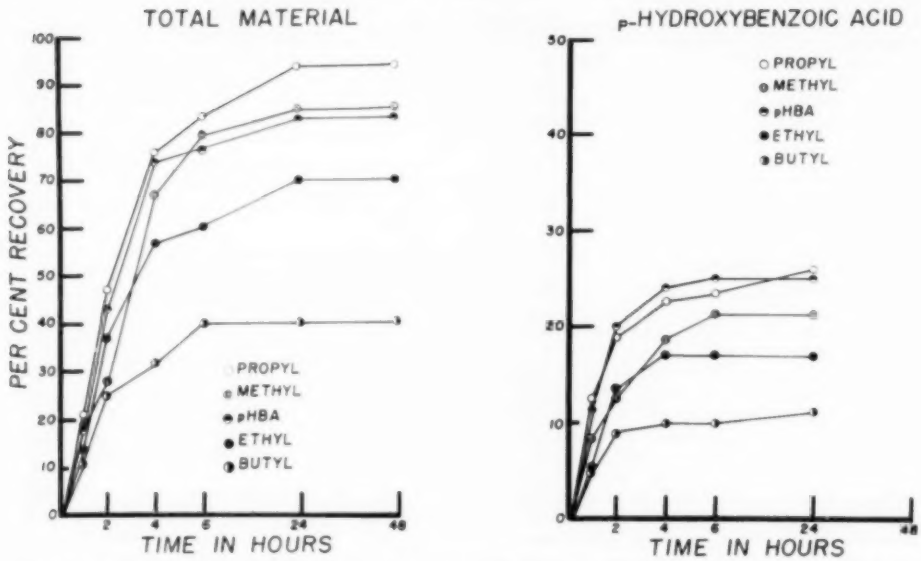


Fig. 1.—Recovery of products of metabolic alteration of *p*-hydroxybenzoic acid and its esters from the urine of dogs given 50 mg./Kg. of each drug intravenously. Each curve represents an average of at least 3 experiments.

following intravenous injections. Significant levels were found only in the plasma of some dogs a few hours after oral administration. The amount of free *p*-hydroxybenzoate excreted never exceeds 0.5% regardless of the method of dosage. The bulk of the "Total" material seems to be excreted in the conjugated form. This conjugation may take place before or after hydrolysis, and the biochemical methods employed cannot distinguish between the two metabolic routes. However, rapid hydrolysis of all but the butyl ester linkages is indicated since after intravenous doses of the compounds practically as much free PHBA is excreted as when the acid itself is injected.

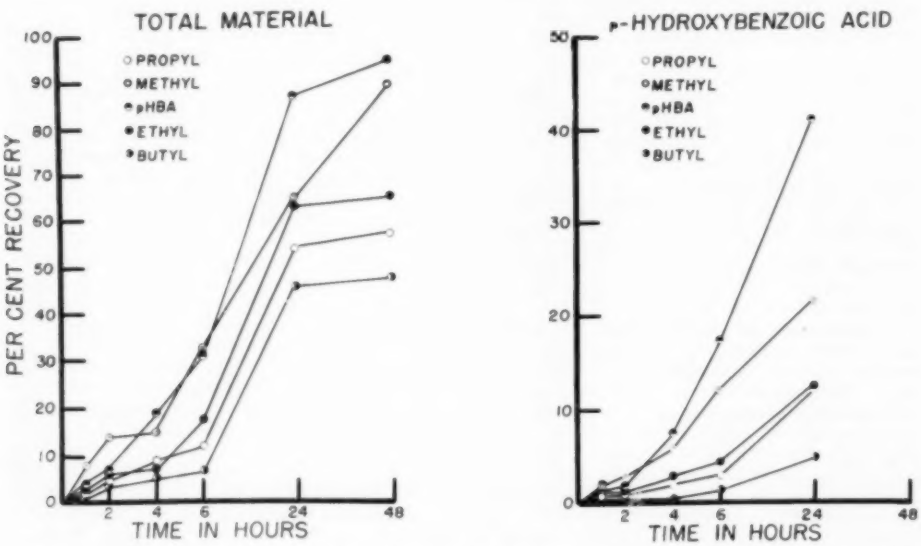


Fig. 2—Recovery of products of metabolic alteration of *p*-hydroxybenzoic acid and its esters from the urine of dogs given 1.0 Gm./Kg. of each drug by mouth. Each curve represents an average of at least 3 experiments.

TABLE I.—SUMMARY OF ABSORPTION AND EXCRETION OF PARAHYDROXYBENZOIC ACID AND ITS ESTERS

Drug	Time	Average of Three Dogs Given 1.0 Gm./Kg. Orally of Each Compound: γ/cc. Plasma Levels — Parasept	Free PHBA	Total	Dose Excreted (Accumulative), % — Parasept	Free PHBA	Gluc. Acid Conjugate	Total	Average of Three Dogs Given 50 γ/Kg. I. v. of Each Compound: γ/cc. Plasma Levels — Parasept	Free PHBA	Total	Dose Excreted (Accumulative), % — Parasept	Free PHBA	Gluc. Acid Conjugate	Total
PHBA	5 min.	..	..	..	..	..	..	..	..	112	122	..	..	..	..
	15 min.	..	46	59	..	..	..	..	..	80	100	..	..	..	..
	30 min.	..	..	..	..	..	..	..	..	59	93	..	..	..	..
	1 hr.	..	249	260	..	1.7	.2	2.0	..	28	73	..	5.5	3.8	18.0
	2 hr.	..	399	420	..	2.0	1.1	6.0	..	1.3	56	..	20.0	18.2	43.1
	4 hr.	..	603	703	..	7.6	4.2	19.1	..	0	13	..	24.0	18.2	74.2
	6 hr.	..	660	660	..	17.8	8.2	30.3	..	0	6	..	25.0	18.2	80.3
	24 hr.	..	4	10	..	40.1	30.0	87.1	..	0	0	..	25.0	18.2	83.0
	48 hr.	..	0	4	..	40.1	30.0	94.0	..	0	0	..	25.0	18.2	83.0
Methyl *p*-hydroxybenzoate	5 min.	..	..	..	..	..	..	..	0	82	115	..	..	..	..
	15 min.	0	15	17	..	..	..	..	0	68	120	..	..	..	..
	30 min.	..	..	..	..	..	..	..	0	48	115	..	..	..	..
	1 hr.	0	110	273	0.003	0.7	0.5	8.1	0	19	113	0.03	8.4	4.3	14.1
	2 hr.	0	293	437	0.005	2.3	2.1	14.4	0	12	61	0.03	12.4	9.5	28.6
	4 hr.	0	553	827	0.005	6.2	4.9	15.5	0	4	27	0.08	18.1	28.5	67.3
	6 hr.	0	630	867	0.014	11.0	28.8	32.0	0	0	12	0.10	20.6	28.9	80.0
	24 hr.	0	19	77	0.014	21.2	33.1	66.1	0	0	0	0.58	20.6	32.0	85.0
	48 hr.	0	0	3	0.014	21.3	35.1	89.0	0	0	0	0.58	20.6	32.0	85.0
Ethyl *p*-hydroxybenzoate	5 min.	..	..	..	..	..	..	..	5	51	85	..	..	..	..
	15 min.	2.3	4.3	32	..	..	..	..	5	62	71	..	..	..	..
	30 min.	..	..	..	..	..	..	..	0	16	66	..	..	..	..
	1 hr.	0	99	134	0.001	0.31	0.26	2.0	0	12	54	0.03	5.7	2.8	14.0
	2 hr.	7.0	130	228	0.005	1.5	1.1	4.5	0	3	36	0.24	13.0	9.2	37.0
	4 hr.	0	375	480	0.005	3.1	2.4	7.4	0	0	30	0.34	17.0	14.0	56.0
	6 hr.	0	427	648	0.006	4.5	7.7	18.5	0	0	3	0.34	17.0	15.0	61.0
	24 hr.	0	0	17	0.034	12.5	35.5	64.0	0	0	0	0.34	17.0	15.0	70.0
	48 hr.	0	0	0	0.034	12.3	32.5	66.0	0	0	0	0.34	17.0	15.0	70.0
Propyl *p*-hydroxybenzoate	5 min.	..	..	..	..	..	..	..	3	85	93	..	..	..	..
	15 min.	2	5.5	16	..	..	..	..	0	62	101	..	..	..	..
	30 min.	..	..	..	..	..	..	..	0	47	83	..	..	..	..
	1 hr.	0	32	79	0.003	0.09	0.11	0.37	0	17	49	0.01	12.5	7.1	20.3
	2 hr.	0	48	142	0.006	0.81	0.68	2.9	0	5	32	0.06	19.0	11.6	47.0
	4 hr.	0	95	232	0.001	2.3	1.8	9.0	0	0	13	0.06	23.0	17.5	75.5
	6 hr.	0	205	370	0.006	3.1	3.0	12.2	0	0	0.3	0.10	23.6	19.7	83.7
	24 hr.	0	13	43	0.042	12.1	12.0	53.2	0	0	0	0.10	26.0	22.6	94.0
	48 hr.	0	0	0	0.042	12.7	16.5	57.6	0	0	0	0.10	26.0	22.6	94.0
Butyl *p*-hydroxybenzoate	5 min.	..	..	..	..	..	..	..	0	47	64	..	..	..	..
	15 min.	5	0	0.7	..	..	..	..	0	39	54	..	..	..	..
	30 min.	..	..	..	..	..	..	..	0	26	36	..	..	..	..
	1 hr.	14	0	15	0.002	0.0	0.10	0.06	0	12	26	0.20	5.0	17.0	11.0
	2 hr.	3	1.3	34	0.018	0.09	0.40	0.87	0	5	14	0.30	9.0	17.0	26.3
	4 hr.	0	5.5	119	0.021	0.32	1.9	2.6	0	1	7	0.31	10.1	19.3	37.1
	6 hr.	0	15	141	0.075	1.05	4.3	6.9	0	0	1	0.31	10.1	20.1	40.1
	24 hr.	0	1.7	25	0.47	4.9	27.5	47.0	0	0	0	0.31	11.3	20.1	40.1
	48 hr.	0	0	0	0.54	4.9	27.5	48.2	0	0	0	0.31	11.3	20.1	40.1

After intravenous administration, the values for free PHBA and "Total" material in the plasma are similar, but the "Total" values remain high for a longer period of time. From this it would seem that the first detoxication mechanism called upon is one of hydrolysis. Peak "Total" concentrations range from 70–110 γ/cc. depending on the drug, and all fractions are gone from the plasma after six hours. After oral doses, levels are still detectable twenty-four hours after ingestion. Higher peak "Total" concentrations (400–600 γ/cc.) are obtained which appear six hours after feeding except in the case of butyl ester where the peak concentration at six hours is only 150 γ/cc. Extremely low free PHBA values are found in the plasma after oral administration of butyl *p*-hydroxybenzoate. This fact coupled with the smaller amounts of free PHBA excreted, again indicates a possible lowered hydrolysis rate of the butyl compound.

A study in which 70 mg./Kg. of methyl *p*-hydroxybenzoate was given orally to a fasted man revealed that the drug is handled similarly in human metabolism. No detectable ester was found in the plasma or urine. Fifty per cent of the administered dose was recovered from the urine after twelve hours as "Total" material of which 11% was accounted for as free PHBA.

No accumulation of products was noted in dogs which had been given 1.0 Gm./Kg. of methyl or propyl orally every day for a period of one year. The rate of urinary excretion in dogs had increased to such an extent that after twenty-four hours, 96% of the dose was recovered in the urine whereas in dogs given a single oral dose, it is not until forty-eight hours that the entire amount is accounted for. Plasma curves were almost identical in both these cases with the chronic dog's peak level appearing earlier.

When a paste of hydrophilic ointment to which 10% of methyl and propyl ester had been added, was applied to the skin on the back of white rabbits, no noticeable irritation was detected after forty-eight hours, and analysis of the kidney after sacrificing the animal revealed no ester or degradation product present.

DISTRIBUTION OF PHBA ACID AND ITS ESTERS

An experiment was designed to study the distribution of PHBA and its esters in animal tissues. Dogs were infused intravenously at the rate of 2 mg./Kg./min. until a total of 100 mg./Kg. of the compound under study was injected. When 95 mg./Kg. had been given, blood samples were taken. The dog was then killed with intravenous air and the tissues immediately isolated. The organs were cut up, mixed with an equal quantity by weight of saline in a Waring Blendor, and finely ground in a tissue grinder until the mash could be drawn through a syringe.

Analyses were made on the various tissues and the plasma to determine the levels of PHBA, ester, and "Total" material present at the time of death. The results are shown in Table II. Appreciable ester is found only in the brain, spleen, and pancreas. High "Total" concentrations are found in the liver and kidney, and high concentrations of free PHBA are detected in the kidney. Other than these instances, levels in the various organs are below plasma levels. Apparently this rate of administration is slow enough to overcome most enzymatic differences in the handling of these compounds, for other than higher ester concentrations, the butyl compound does not appreciably differ from the others.

In vitro studies of normal tissue mashes mixed

TABLE III.—RATE OF HYDROLYSIS OF ESTERS OF PARA-HYDROXYBENZOIC ACID BY DOG TISSUE MASHES. DRUG CONCENTRATION 100γ/CC., TISSUE DILUTED 1:200 AND REACTION CARRIED OUT AT ROOM TEMPERATURE

Tissue	Time, Min.	Compound Hydrolyzed, %: Methyl Ester	Ethyl Ester	Propyl Ester	Butyl Ester
Liver	3	100	96	100	64
	30	100	96	100	100
	60	100	96	100	100
Brain	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Kidney	3	72	60	18	14
	30	100	100	100	74
	60	100	100	100	100
Muscle	3	0	0	0	0
	30	16	16	0	0
	60	18	16	8	1.4
Spleen	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Pancreas	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Plasma	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Whole blood	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0

TABLE II.—DISTRIBUTION OF PARAHYDROXYBENZOIC ACID, ITS ESTERS, AND METABOLIC PRODUCTS IN TISSUES OF DOGS EACH COMPOUND INFUSED INTRAVENOUSLY AT A RATE OF 2 MG./KG./MIN. FOR FIFTY MINUTES

	Concentration in Tissues (γ per Gm. or ml.)														
	Concentration of PHBA					Concentration of Ester					Concentration of "Total"				
Tissues	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA
Liver	28	19	28	74	56	0	0	0	0	.	240	245	280	520	540
Kidney	186	116	150	190	192	0	0	0	0	.	520	330	330	520	540
Brain	0	0	3	14	20	50	23	30	10	.	36	25	36	32	64
Spleen	14	35	20	36	64	32	0	0	6	.	76	53	80	56	108
Pancreas	2	10	16	..	30	70	0	16	..	.	80	50	132	..	124
Muscle	9	23	18	12	24	0	0	0	0	.	30	48	120	34	42
Plasma	72	72	47	155	225	25	5	0	0	..	193	275	177	265	300

with the various esters revealed that esterases in liver and kidney were extremely efficient in hydrolyzing the esters to free PHBA. The liver mashes were capable of 100% hydrolysis after three minutes of contact with all the esters except butyl in which case thirty to sixty minutes of contact was necessary. Muscle mash was capable of this hydrolysis to a small degree while the other tissues, plasma, and whole blood showed no *in vitro* ability to break the ester linkage. The liver, having low free PHBA levels in the intact animal, presumably conjugates or otherwise metabolizes most of its free PHBA to further degradation products.

DISCUSSION

From the studies reported above it is apparent that *p*-hydroxybenzoic acid and its esters are efficiently absorbed from the gastrointestinal tract and undergo rapid metabolic alteration. The esters in particular are rapidly hydrolyzed by esterases found in the liver and kidney. That this enzyme differs from that which ordinarily splits procaine is evident from the fact that procaine esterase is abundantly present in plasma, whereas the esters of *p*-hydroxybenzoic acid are not hydrolyzed by plasma or whole blood. Similarly it is unlikely that choline esterase is responsible for the hydrolysis since it was shown that the administration of physostigmine in highest tolerated doses had no effect on the rate of disappearance of the esters of *p*-hydroxybenzoic acid from the blood.

SUMMARY

1. Appropriate chemical methods have been devised for the quantitative analysis of *p*-hydroxybenzoic acid and its esters in tissues and biological fluids. With suitable modifications, this procedure may be employed to determine "Total" material, that is, ester plus free PHBA plus all metabolic products in which the phenolic group is not destroyed.

2. By means of these methods a comparative study has been made of the plasma levels and urinary excretion of dogs given 1.0 Gm./Kg. orally and 50 mg./Kg. intravenously of methyl, propyl, and butyl ester as well as PHBA itself. Per cent recoveries from the urine of these animals, regardless of the method of administration, ranged from 60–95 per cent except in the case of butyl ester where the recoveries were approximately 40 per cent. These results seem to indicate that PHBA and the esters are well absorbed after oral administration.

3. Only rarely do the plasma ester levels reach a significant value even immediately following intravenous injection. Highest ester levels are encountered after the oral administration of the butyl derivative. On the other hand, the high plasma levels and urinary output of free PHBA and conjugated products in dogs having received the various esters indicate that hydrolysis of the ester linkage and metabolic conjugation by the body constitute the chief paths of metabolic alteration. However, when the butyl derivative is administered rapidly there appear to be other pathways in addition which account for poor recovery.

4. Studies in which tissue mashes were allowed to incubate with the *p*-hydroxybenzoates revealed rapid *in vitro* enzymatic hydrolysis of the compounds by liver and kidney. The butyl ester was found to be the least easily hydrolyzed of the series.

5. Topical application of ointments which contained 10 per cent methyl and 10 per cent propyl *p*-hydroxybenzoate to the shaved backs of white rabbits produced no irritation after forty-eight hours of contact. Upon sacrificing the animals, no drug concentration was detected in the kidneys.

REFERENCES

(1) Emerson, E., *J. Org. Chem.*, **8**, 417(1943).
(2) Williams, R. T., "Detoxication Mechanisms," John Wiley & Sons, Inc., New York, N. Y., 1947, p. 104.
(3) Maughan, G. B., Browne J. S. L., and Evelyn K., *Biol. Chem.*, **126**, 567(1938).

A Note on the Determination of Some Alkali Salts of Organic Acids by Ion Exchange*

By SEYMOUR M. BLAUG

The present official method of analysis for preparations containing alkali salts of organic acids is time consuming. A cation exchange resin was used to convert the salts to the corresponding free acid which was then titrated directly with sodium hydroxide. The method was applied to four parenteral preparations, i. e., anticoagulant sodium citrate solution, anticoagulant acid citrate dextrose solution, sodium lactate injection, and sodium salicylate ampuls. The citric acid in A. C. D. solution was determined by direct titration before passing the solution through the resin column. Dextrose does not interfere in the ion exchange assay procedure. The ion exchange assay procedure is rapid, with an accuracy equal to or better than that achieved by the official method.

THE OFFICIAL ASSAY of alkali salts of organic acids (1) involves carbonizing the salt, then digesting the carbonized mass with sulfuric acid. The residual acid is titrated with sodium hydroxide. Although this method is a satisfactory assay procedure, it has several disadvantages. The number of manipulations involved not only makes the procedure tedious but allows for more sources of error.

It was the purpose of this paper to develop a rapid, accurate method for the determination of alkali salts of organic acids which could be applied to the control of parenteral solutions made in our laboratory.

In the procedure developed in this study, the salts are determined by the use of a strongly acidic cation exchange resin. When a solution of an alkali salt of an organic acid is passed through a column containing the resin, the cations of the solution are replaced by hydrogen ions, making possible a direct titration of the total acid with standard sodium hydroxide.

EXPERIMENTAL

Materials.—Of the several cation exchange resins investigated, Amberlite® IR-120[1] gave the most consistent recoveries. The resin was obtained in the analytical grade which is supplied in the hydrogen form.

The resin columns were prepared by slurring 10 Gm. of wet resin into columns having the following characteristics: 20 cm. in length, 1 cm. in diameter, and one end constricted to a diameter of 0.5 cm. The constricted end was packed with glass wool, the glass wool serving as a base for the resin. The rate of flow was adjusted to 0.15 cc./cc. of resin/min. by means of a Teflon® tube and Hoffman clamp attached to the constricted end of the column. The resin was washed with several portions of distilled water. A column so prepared can be used several times without regeneration.

The sodium citrate, citric acid, dextrose, sodium lactate, and sodium salicylate used in this study were commercial samples of U. S. P. or N. F. quality. The parenteral preparations used were prepared in our manufacturing laboratory.

Determination of Salts

Sodium Citrate.—Approximately 0.5 milliequivalent of sodium citrate was accurately weighed and dissolved in 10 cc. of distilled water and the solution passed through a column of Amberlite® IR-120. The column was washed with 4 × 10 cc. portions of distilled water. The free acid present in the eluate was determined by titration with 0.1 *N* sodium hydroxide using two drops of phenolphthalein as indicator.

Fifty cc. of water was passed through the same column and titrated with 0.1 *N* sodium hydroxide. In this way the completeness of the washings could be controlled by determination of the blank value immediately after the analysis.

Sodium Lactate.—Since sodium lactate is supplied as a 60 per cent solution, approximately 1 Gm. of the solution was accurately weighed in a 100-cc. volumetric flask and diluted to 100 cc. with water. Ten cc. of this solution (equivalent to approximately 0.5 milliequivalent of sodium lactate) was then treated in the same manner as described for sodium citrate.

Sodium Salicylate.—Sodium salicylate was determined in the same manner as described for sodium citrate, using 75 per cent ethanol as the solvent instead of water. This was necessary in order to achieve quantitative elution from the resin column since the salicylic acid formed is not very soluble in water.

For purposes of comparison all three salts were assayed by the official method described under the individual monographs in U. S. P. XV. Results of the official method and the ion exchange method are tabulated in Table I.

TABLE I.—ANALYSIS OF SALTS

Salt	Wt. Sample, Gm.	Recovered, % U. S. P.	Recovered, % Ion Exchange
Sodium citrate	0.1459	98.11	99.28
Sodium lactate[a]	0.1044	55.45	56.13
Sodium salicylate	0.2002	98.20	99.21

[a] Sample labeled "approximately 60 per cent."

* Received August 20, 1955, from the State University of Iowa, College of Pharmacy, Iowa City.

[1] Rohm and Haas Company, Philadelphia, Pa.

TABLE II.—ANALYSIS OF PARENTERAL PREPARATIONS USING AMBERLITE® IR-120

Preparation	Theoretical Content	Recovered, Gm.	Labeled Amt., %
Anticoagulant sodium citrate solution	40 Gm./1000 cc.	39.80	99.50
A. C. D. solution	13.2 Gm./1000 cc.	13.05	98.86
Sodium lactate injection	11.2 Gm./40 cc.	11.40	101.78
Sodium salicylate ampul	2 Gm./10 cc.	1.98	99.00

Determination of Salts in Parenteral Solutions

Anticoagulant Sodium Citrate Solution.—The theoretical content of sodium citrate in anticoagulant sodium citrate solution is 40 Gm./1000 cc. A 20-cc. aliquot was withdrawn from a liter bottle of anticoagulant sodium citrate solution and diluted to 100 cc. Twenty cc. of this dilution was passed through a column of Amberlite® IR-120 and treated as described previously under the determination of salts.

Anticoagulant Acid Citrate Dextrose Solution.—A. C. D. solution contains free citric acid and dextrose in addition to sodium citrate. The citric acid was determined by titrating an aliquot of the A. C. D. solution with sodium hydroxide as described in the U. S. P. monograph. Sodium citrate and dextrose do not interfere. A 10-cc. aliquot was withdrawn from a liter bottle of A. C. D. solution and treated by the ion exchange method previously outlined. The amount of sodium citrate present was calculated by subtracting the number of cc. due to the free citric acid from the total number of cc. consumed in the ion exchange procedure. Dextrose did not interfere with the ion exchange procedure.

Sodium Lactate Injection.—Sodium lactate injection is prepared in 40-cc. vials containing 11.2 Gm./40 cc. A 10-cc. aliquot was withdrawn from such a vial and diluted to 100 cc. with water. Ten cc. of this dilution was passed through a column of Amberlite® IR-120. The column was eluted and the eluate titrated as described previously.

Sodium Salicylate Ampuls.—Sodium salicylate ampuls are prepared to contain 2 Gm. of sodium salicylate in a 10-cc. ampul. The contents of 4 ampuls were washed into a 500-cc. volumetric flask with 75 per cent ethanol, and diluted to volume with the solvent. A 10-cc. aliquot was withdrawn and passed through the resin column. The column was eluted with 4 × 10 cc. portions of 75 per cent ethanol and the eluate titrated with 0.1 *N* sodium hydroxide.

Results of the assay of parenteral solutions are tabulated in Table II.

SUMMARY

A method for the determination of some alkali salts of organic acids by the exchange of ions has been described. The method is rapid, reproducible, and applicable to the assay of official preparations containing these salts.

REFERENCES

(1) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 892.

Book Notices

Methoden der organischen Chemie (Houben-Weyl). By EUGENE MULLER. Vol. IX Schwefel-, Selen-, Tellur- Verbindungen. Georg Thieme Verlag, Stuttgart, 1955. xix + 1337 pp. 19 x 26 cm. Price DM 196.20.

This is the sixth of a projected series of fourteen volumes of the monumental new edition of *Methoden der organischen Chemie*. The first of the series was published in 1952 and the design of the new edition was previously described [THIS JOURNAL, **42**, 643(1953)]. When the project was started, it was expected that each of the several volumes would be restricted to approximately 700 pages and that the cost of each would be approximately DM 100. It has not been possible to adhere to this original limitation on the number of pages in each volume. The most recent volume, covering the sulfur, selenium, and tellurium compounds, despite effort to condense and to select critically the available material, requires more than 1,300 pages. This is understandable because of the increasing importance of sulfur containing organic compounds in the manufacture of dyes, pharmaceuticals, detergents, and other industrial chemicals. The selenium and tellurium organic compounds, while possibly of lesser significance, have also been covered very adequately and quite comprehensively.

The book is logically divided into three parts and the major portion is devoted to 24 chapters on the sulfur compounds. The selenium and tellurium compounds are covered in 17 chapters.

A conception of the scope and arrangement of Volume IX of *Houben-Weyl* can best be indicated by citing some of the main subjects of the several chapters. These are as follows:

Mercaptans and thiophenols; alkyl-hydrogen-polysulfides; disulfides; polysulfides; thioethers; ethylene sulfides; sulfonium compounds; mercaptals and mercaptols; sulfoxides and sulfinimines; sulfones and sulfoximines; aliphatic sulfuric acids and their derivatives; aliphatic and aromatic sulfinic acids; aliphatic sulfonic acids and their derivatives; aromatic sulfonic acid anhydrides; aromatic sulfohalides; functional nitrogen derivatives of aryl sulfonic acids; aromatic sulfonic acid esters; thiosulfonic and thiosulfuric acid esters; thioaldehydes and ketones; thioacids and their derivatives; and sulfur-containing carbonic acid derivatives.

There is scarcely a chapter in the entire volume which is not of considerable pharmaceutical interest. The preparative methods and other subjects are sufficiently comprehensive so that the necessity of consulting the original literature is greatly minimized. Despite this, however, the book is adequately documented throughout with references to original articles, extending through part of 1955. The value of the book is enhanced by a chapter on the nomenclature of the sulfur compounds, an author index, and a subject index. The printing and binding are of excellent and durable quality and the editors, the contributors, and the publisher are to be congratulated upon producing another volume in a most important series of books of interest to organic chemists.

A Short History of Medicine. By Erwin H. Ackerknecht. The Ronald Press, New York, 1956. xviii + 258 pp. 14 x 21 cm. Illustrated. Price $4.50.

Many laymen will be fascinated by this history and medical men will find it a quick way to acquaint themselves on the development and trends in medicine from the early gropings of primitive man to the present day. There are many illustrations, a good index, and, what is more informative than the usual bibliography, a section on "Suggestions for Further Reading." This is correlated to each chapter to facilitate the pursuit of a particular facet of medical science or of a particular culture. It is a very interesting book.

The Year Book of Drug Therapy (1955–1956 Year Book Series). Edited by Harry Beckman. The Year Book Publishers, Inc., Chicago, 1956. 560 pp. 14 x 20 cm. Price $6.

This volume starts with cogent observations by Dr. Beckman on the highlights of developments in the various medical classifications into which the book is divided. The text contains the excellent selection of abstracts of papers published in the divisions which are arranged in alphabetical sequence from allergy to venereology. The extensive coverage is indicated by the chapter headings: allergy, antibiotics and sulfonamides, cardiovascular diseases, dermatology, endocrinology, gastroenterology, hematology, internal medicine, neuropsychiatry, obstetrics and gynecology, ophthalmology, otorhinolaryngology, pediatrics, surgery, and venereology. No pharmacy reference library should overlook this series of annual reviews.

BOOKS RECEIVED

Epitome of the Pharmacopeia of the United States and the National Formulary with Comments. 10th ed. Issued under the direction and supervision of the Council on Pharmacy and Chemistry of the American Medical Association. J. P. Lippincott Company, Philadelphia, 1955. xiii + + 322 pp. 13 x 19.2 cm. Price $3.

A Textbook of Pharmacognosy. By N. M. Ferguson. The Macmillan Company, New York, 1956. vii + 374 pp. 14 x 23.5 cm. Price $7.

Blood Group Substances—Their Chemistry and Immunochemistry. By Elvin A. Kabat. Academic Press, Inc., New York, 1956. ix + 33 pp. 16 x 23.6 cm. Price $8.

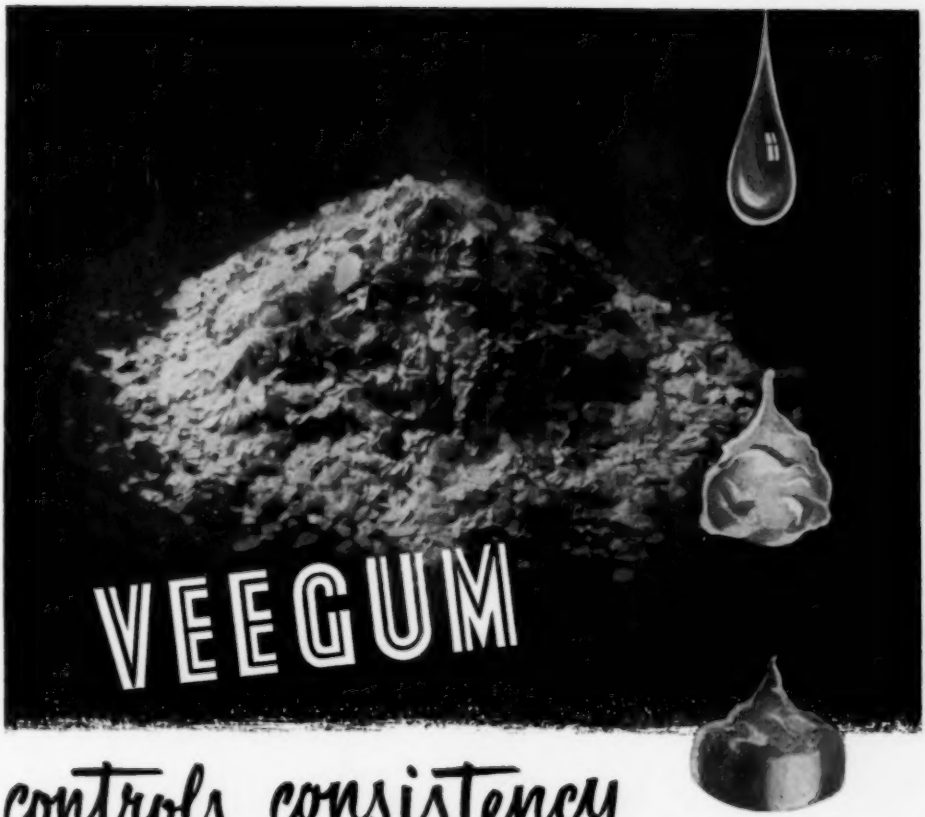
The Mathematics of Physics and Chemistry. 2nd ed. By Henry Margenau and George M. Murphy. D. Van Nostrand Company, Inc., Princeton, N. J., 1956. xii + 604 pp. 16 x 23.5 cm. Price $7.95.

Experimental Tuberculosis. Bacillus and Host. Addendum on Experimental Leprosy. G. E. Wolstenholme and Margaret P. Cameron, Editors for the Ciba Foundation. Little, Brown and Company, Boston, 1955. xii + 396 pp. 14 × 21 cm. Price $9.

The Complete Book of First Aid. By John Henderson. Duell, Sloan and Pearce, New York—Little, Brown and Company, Boston, 1955 341 pp. 13 x 22 cm. Price $3.50.

Calendar of the Pharmaceutical Society of Great Britain 1955–1956. Pharmaceutical Press, London, 1955. (Rittenhouse Bookstore, Philadelphia.) 296 pp. 14.2 x 22 cm. Price 15*s.* plus 1*s.* postage.

Biochemistry for Medical Students. 6th ed. By W. V. Thorpe. J. B. Lippincott Company, Philadelphia, 1956. viii + 542 pp. 14.5 x 21 cm. Price $6.75.



controls consistency

Veegum is a unique suspending, emulsifying and binding agent developed to enhance the physical qualities of your liquids, lotions, pastes, and tablets. *Inorganic*, Veegum suspends at lower viscosities than ordinary gums — *thixotropic*, it gives added long-term stability — *thickening slightly with heat*, it maintains product consistency at above-normal storage temperatures.

Aqueous dispersions of nontoxic, white Veegum are compatible with most oils, fats, waxes, and solvents over a wide range of pH. Try Veegum in your own laboratory. You will find it a versatile, high-quality physical conditioner for all your pharmaceuticals.

SEND FOR THE VEEGUM STORY

R. T. VANDERBILT CO.

SPECIALTIES DEPARTMENT

230 PARK AVENUE NEW YORK 17, N. Y.

Please send ☐ Veegum Bulletin B53 ☐ Sample of Veegum
☐ Information on using Veegum for:

APPLICATION_____

NAME_____

POSITION_____

(Please attach to or write on your company letterhead)

THE USE OF *Sealva*

(Hermetically Sealed dry flavors)

IN PHARMACEUTICALS

The problems of improving palatability in dry medicinals can be greatly simplified through the use of Sealva, "sealed-in" flavors.

The complete and varied line of ALVA liquid flavoring materials, developed for use in pharmaceuticals and proprietaries, can be supplied in the form of fine-grain inert powder (actually droplets of flavoring materials, natural or artificial, hermetically sealed in an edible gum film). This process protects the flavor from deterioration by evaporation, oxidation or chemical reaction with other components.

Sealva flavors offer an especial advantage for medicinals in powder, grain, flake, compressed tablet and oil suspension form.

Samples and technical data are available at your request and the facilities of the Alva Flavor Laboratory are ready to help with recommendations on any specific palatability problem.

VAN AMERINGEN-HAEBLER, INC.

521 WEST 57th STREET, NEW YORK 19, N. Y.

Alva FLAVORS